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Motile vacuolar systems in plant cells: a review and analytical study.

Thomas P. Wilson.

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Motile vacuolar systems in plant cells: a review and analytical study.

submitted by

Thomas P. Wilson, B.Sc.

in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Biology

Thesis supervisors

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Abstract

Living plant cells possess a motile vacuolar system (MVS) separate from a larger, central vacuole. These elongated 1-6 μm diameter vacuoles vesiculate during chemical fixation (e.g. with glutaraldehyde and/or osmium tetroxide) or cellular damage. Therefore, intact MVS cannot be observed in tissue prepared for electron microscopy by conventional methods. Although the MVS is extensive, it is inconsistently identified in the literature, due to workers interpreting similar observations of living cells differently. For example, MVS has been variously labelled as a vacuolar system, endoplasmic reticulum (ER), and actin microfilaments. A critical review shows that this confusion is partly due to the inherent ambiguity of phase contrast and differential interference contrast (DIC) photomicrographs. An appreciation of early descriptions of vacuoles and ER, and attention to the three-dimensional geometry of the membrane system clears up this ambiguity. Correct interpretation of the system's morphology has permitted a mathematical model that predicts the size and number of vesicles resulting from MVS vesiculation. However, mathematical modelling does not reveal the physicochemical cause behind vesiculation. Physical modelling using myelin forms has shown that fixatives do not directly cause vesiculation; instead, changes in the cytoplasmic ionic environment during cell death may lead to vesiculation. Changes in calcium distribution (as shown by chlortetracycline) were found during fixation of onion epidermal cells. Since problems have historically existed with distinguishing and studying the MVS, I exposed the MVS in onion (*Allium cepa* L.) bulb scale epidermal and tomato (*Lycopersicon esculentum* Mill.) petiolar hair cells to different vital dyes with a view to using staining patterns to help discriminate MVS from surrounding cytoplasm. Neutral red (typically a vacuolar stain) showed that the MVS and central vacuole were separate in onion cells, uranin (a

general protein stain) was useful in distinguishing cytoplasmic from vacuolar space, and staining with DiOC₆ (which labels ER) showed that ER, unlike MVS, survives conventional chemical fixation. Through centrifugation of living hair cells, it was found that the MVS is denser than the larger central vacuole, and is among the first organelles to return to pre-centrifugation distribution. Actin labelling (with rhodamine-phalloidin) and treatment with actin-disrupting drugs have shown that vacuolar movement, distribution, and orientation are actin-dependent.

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Abbreviations:

DIC = differential interference contrast

DiOC₆ = 1,3-dihexyloxacarbocyanine

ER = endoplasmic reticulum

MVS = motile vacuolar system

VE-DIC = video enhanced DIC

General Introduction

Much of our current knowledge of cell biology comes from the study of dead cells. The licence to create theories describing the structure of living cells with evidence procured from dead specimens is derived from the faith that fixation preserves cell structure with fidelity. However, conventional chemical fixation procedures produce dramatic reorganization of plant cytoplasm. This was convincingly demonstrated by Mersey (1977) and Mersey and McCully (1978). Mersey showed how fixation 'e.g. with glutaraldehyde) disrupted an extensive membrane system in the cells of tomato petiolar hairs. The tomato petiolar hairs (Fig. 1) were an ideal system for observing fixation, due to their large size and optically transparent walls. When these cells are viewed with phase contrast optics (Fig. 2), one is primarily impressed by their dynamic cytoplasmic streaming which appears well-directed and controlled. The nucleus remains stationary, as do the chloroplasts which are mostly attached to a non-motile cortical cytoplasmic layer, but mitochondria and some chloroplasts are carried in the cytoplasmic streams. Also moving with the motile cytoplasm is an extensive system of elongated vacuoles (each vacuole about 1-6 μm in diameter, see Fig. 3). This is the membrane system which Mersey disrupted with chemical fixation, and named the pleomorphic canalicular system (PCS). The system may also be found in other plant cell types; for example, Wilson *et al.* (1992) demonstrate its presence in cortical cells of pea (*Pisum sativum* L.) root, protoplasts from tobacco (*Nicotiana tabacum* L.) stem tissue, snowberry (*Symphoricarpos albus* Blake) mesocarp cells, and onion (*Allium cepa* L.) bulb scale epidermal cells (onion cells are shown in Fig. 4). Although the name proposed by Mersey (1977) (pleomorphic canalicular system) does give some indication of the system's morphology and dynamic nature, I believe there are short-

comings to this label. First, I feel that the motility of the system is a more important property than its fluid character. The system's motility is responsible for its distribution in the cell, and may be important in any proposed function. Second, although the term 'canalicular' is sufficiently noncommittal as to avoid potential conflict with other identifications in the literature, it does not give any indication of the size of the system's constituents relative to other accepted membrane systems. The membrane forms making up the system have the appearance and size typically assigned to vacuoles (this is discussed more fully below). Identifying the system as a collection of vacuoles gives a more specific image of the size and character of its component parts. A new name, the motile vacuolar system (MVS), gives a clearly indicates the system's dynamic organization, and unequivocally identifies the system as vacuolar. One last point about naming. I have consistently met with the opinion that 'pleomorphic canalicular system' is too difficult a name to cope with. This might be due to the name's inherent vagueness (what size are these channels and how are they pleomorphic), or to the unfamiliarity of the words. 'Motile vacuolar system' clearly refers to a group of vacuoles moving around within a cell, and is a term that easily fits into established mental glossaries.

Fig.1 Live tomato petiolar hair photographed through an Olympus SZH stereo microscope with a DH 1X PLAN objective and ring light source (60X).

Figs. 2 and 3. Live basal cell in a detached tomato petiolar hair mounted in distilled water under a cover-slip. Improvised phase contrast optics (constructed according to Baker, 1957). In Fig. 2 the cell wall (W) delimits the cytoplasm which streams towards and away from the central nucleus (N). Chloroplasts (C) maintain mostly stationary positions in a non-motile cortical cytoplasmic layer, although some may move with the interior streaming cytoplasm. Fine dark lines visible in the cytoplasm (bracketed by arrows) are the boundaries of of long motile vacuoles (660X). Figure 3 shows a sheet of peripheral cytoplasm containing some of the motile vacuolar system (MVS). The vacuoles are more phase-bright than the surrounding cytoplasm. The size of the individual vacuoles can be compared to chloroplasts (C) (1320X).

Fig. 4. Onion bulbscale epidermal cell viewed with phase-contrast optics. The phase-bright vacuoles of the MVS (arrow) are clearly visible. Part of the MVS is always closely associated with the nucleus (N) , both in the hair cells and the onion epidermal cells (1200X).

A vacuole is generally defined as a fluid-filled cavity within the cytoplasm, containing a variety of dissolved substances (usually low molecular weight solutes), separated from the surrounding cytoplasm by a semipermeable unit membrane, the tonoplast. Tonoplast is a general name given to the bounding membrane of any vacuolar space and is not reserved for specific vacuoles (Gunning and Steer, 1975). The appearance of this bounding membrane may vary, depending upon the vacuole. It always has a dark-light-dark tripartite appearance in the transmission electron microscope (TEM) but may be asymmetrical. This means that the dark layer facing the vacuolar space is thicker than the dark layer on the cytoplasmic face (Gunning and Steer, 1975; see their Fig. 17b). This asymmetry is often due to vacuolar contents precipitating during tissue preparation, and these contents can vary even within one cell type (Hardham and McCully, 1982). In freeze-fracture, it is usually found that the cytoplasmic face of the tonoplast has more intramembranous particles than the vacuolar face (Robinson, 1985). Although 'tonoplast' is often used in a general sense to denote "the limiting membrane of the vacuole", it is biochemically distinct (e.g. tonoplast-specific ATPases; Robinson, 1985). Therefore, in the absence of biochemical evidence showing the presence of these membrane markers, the limiting membrane of the MVS will not be referred to as a tonoplast.

Vacuoles may have storage or lytic functions. These functions relate directly to the vacuolar contents. For example, vacuoles may possess a wide variety of hydrolases including proteases, ribonucleases, and phosphatases (Matile, 1978). The storage capacity of vacuoles is well illustrated by their function as protein compartments in seed aleurone cells, and for malate compartmentation in Crassulacean acid metabolism. In transmission electron micrographs vacuoles usually appear as electron transparent regions bounded by a tonoplast. These vacuoles are ubiquitous, and within

any given plant cell (with or without a large central vacuole) one may find a number of small cytoplasmic vacuoles. Problems in identifying a vacuole occur when the radius of the membrane-bound space is similar to that of structures which have alternative names and functions: for example, smooth endoplasmic reticulum (ER) or dictyosome-derived vesicles (ribosome-studded rough ER is easily distinguished). In contrast, biochemical identification of membrane fractions is made reliable by standard membrane markers (e.g. the ER glycoprotein, endoplasmin, Koch *et al.* 1987; or tonoplast ATPases, Robinson, 1985). However, microscopists often base positive identification on structural clues alone, particularly size. Membrane-bound spaces larger than $\sim 0.5\ \mu\text{m}$ in diameter are generally identified as vacuoles (or, if spherical, they may be given a more general name: vesicles). By accepting this size limitation I do not dismiss the presence of vacuoles smaller than $0.5\ \mu\text{m}$. However, I do dismiss the possibility that ER (rough, smooth or otherwise) will frequently be found to have a lumen distended beyond $0.5\ \mu\text{m}$ in diameter. ER is highly variable in form, but is usually identified in electron, fluorescence, or video enhanced microscopy as an extensive anastomosing network with tubules less than $0.4\ \mu\text{m}$ in diameter (Palade and Porter 1954, Terasaki *et al.* 1984, Quader and Schnepf 1986, Hepler *et al.*, 1990). Although vacuoles are thought to derive from Golgi or ER (Marty, 1978; Robinson, 1985), there is a point in this proposed developmental sequence where the structure changes in form and properties such that it becomes vacuolar. I take the position in this thesis that membrane-enclosed spaces with a diameter greater than about $0.5\ \mu\text{m}$ will be grouped under the general term 'vacuole'.

I make this distinction clear from the beginning because of confusion concerning the MVS when Mersey and McCully (1978) published their study of cytological fixation. Their important message was that microscopists should be aware

of fixation artefacts, and that the study of fixation gives the technique of tissue preparation greater analytical power. However, attention was drawn away from this message when critics speculated that Mersey and McCully were viewing ER, since their images resembled those published in some phase contrast investigations of the 1960's (in which the name ER was given to the phase-bright spaces). It seems surprising that the identity of such an extensive and obvious membrane system should be in contention. The dispute indicates that this membrane system might somehow fall outside conventional definitions. The problem lies in the appearance of the system when viewed in the living cell. In phase contrast the vacuoles appear as phase-bright regions separated by phase-dark boundaries (Fig. 3). However, the phase-dark lines between the vacuoles could be viewed as individual filaments separated by cytoplasmic rather than vacuolar space. It is these two possible interpretations of the same image which have caused such confusion in the literature. This confusion could be dispelled by acquiring an historical perspective on the relative morphologies of vacuoles and ER, and then critically examining the literature which attempts to distinguish between the two. Chapter 1 contains an historical analysis of the literature which caused plant biologists to associate the vacuoles viewed by Mersey and McCully (1978) with endoplasmic reticulum.

The presence of a dispute over the identity of this membrane system makes it seem incorrect to include an *a priori* assumption in my choice of the system's name. Although I have already clarified my criterion for distinguishing vacuoles from smaller membrane-enclosed spaces, the name indicates that I have made my decision concerning the two possible interpretations for Fig. 3. That is, the phase bright regions between the dark lines represent vacuolar space, and the lines themselves are intervening cytoplasm. This interpretation follows from Mersey's (1977) work

describing the breakdown of these membranes during cytological fixation. When the cell is fixed, the phase-bright regions in the Fig. 3 break up into spherical vesicles. These vesicles can only arise from the necking and subsequent break-up of fluid cylinders of the appropriate diameter. Filaments or small parallel tubules (which would be represented by the fine dark lines) could not form the observed patterns of vesiculation. Therefore, the vesiculation event is useful for determining the morphology of the original membranes. With this in mind, it would be profitable to further examine vesiculation to discover other underlying properties of the MVS reflected in vesiculation patterns. Two approaches to examine vesiculation will be used here and are presented in Chapter 2. First, a mathematical model will be constructed to describe the geometric consequences of cylinders breaking up into spheres. Physical modelling will also be used to help discover the chemical cause behind vesiculation. Will fixatives vesiculate artificial membrane cylinders, or are other factors involved? Both modelling approaches will point out properties of the intact MVS and the events leading to its breakdown during cytological fixation.

As mentioned above, the confusion over the identity of the MVS indicates a general ignorance of its presence and properties. In fact, I have met with some skepticism over the existence of a secondary vacuolar system separate from a large central vacuole. Many plant biologists have become bamboozled by their own simplified plant cell model, where a plant cell contains a single large central vacuole surrounded by cytoplasm. Often, skepticism arises from a difficulty in making conceptual connections between the MVS seen in phase contrast and the MVS-derived vesicles regularly observed in electron micrographs. Due to the lability of the system with fixation, it may only be studied intact in living specimens. Studying the properties of the MVS in living cells would serve a dual purpose. First, its reaction to different

stains may help to distinguish the MVS from membrane systems (e.g. the ER) which might have a similar appearance when viewed with phase contrast or differential interference contrast (DIC) optics. Specifically, neutral red and uranin (sodium fluorescein) will be used as vacuolar and cytoplasmic stains, respectively; while a fluorochrome found to stain ER, 3-3' dihexyloxacarbocyanine iodide (DiOC₆), will be used to allow comparison between the ER and the MVS in the same cell. Second, it is important to discover how the vacuoles are associated with the surrounding cytoplasm, especially with cytoskeletal components which might power its motility, and pattern its organization within the cell. These MVS-cytoplasmic associations will be examined by observing the response of living cells to centrifugation, and the effect of disrupting either microtubules or actin. Centrifugation has been shown to stratify different cytoplasmic fractions according to their relative densities (Mottier, 1899; Bouck, 1963), and healthy cells will reverse this stratification to return to pre-centrifugation organization. It will be interesting and informative to observe the reorganization of the MVS during this recovery process. Previous work with actin-disrupting drugs has shown that the movement of the MVS vacuoles is actin-dependent (Mersey, 1977). This dependence of vacuolar motility on actin will be re-examined here using a different actin-disrupting drug, cytochalasin D. The effects of actin-disruption will also be observed on cellular recovery following centrifugation. In addition, actin labelling, using rhodamine-phalloidin, will reveal actin organization relative to general MVS distributions and cellular patterning.

Chapter 1. Literature review

Introduction

When Mersey and McCully (1978) published their work describing the effects of cytological fixation on a special membrane system in tomato petiolar hair cells, confusion within the community concerning the identity of the membrane system deflected attention from the study's important message. This confusion in itself is an interesting phenomenon and illustrates how well a particular bit of information is accepted depending upon how that information fits with current models and trends. Within the field of plant cell microscopy, interest in particular structures has shifted according to available observational techniques, and partly under the impetus of new discoveries. In sorting out the confusion over the identity of the MVS one discovers how knowledge contained in older literature becomes less accessible, lost, or transformed. To understand this dispute over the identity of a very obvious cellular structure we must critically examine publications distinguishing between vacuoles and ER. This is best done with some feeling for the historically accepted definitions of these two membrane systems.

In the first half of this century, when workers regularly observed living cells, the plant cell vacuome (a term used to include all vacuolar space in a cell) was known to vary in form and organization. Not only could a cell possess a secondary vacuolar system separate from a larger central vacuole, but a large vacuole could reversibly separate into many smaller vacuoles (Guilliermond, 1933). I will review a sample of this pre-1940 work in order to establish that botanists during this period had a model of

the plant vacuome different from our contemporary model. A plant cell was known to be a dynamic and variable structure, not the static, idealized icon sold to pre-biologists in their first year botany text-books. Vacuoles varied in form and size: they could be long and cylindrical, or isodiametric. However, this intimate familiarity with the living plant cell was lost as the technology of electron microscopy altered our view of plant cytology.

The advent of electron microscopy, more than any other influence, pushed botanists to look primarily at very thin slices of cytoplasm in fixed tissue. The model of the plant cell changed with our observational techniques. Vacuoles became simple, static bags of cellular garbage, while interest in new organelles waxed. During the 1940's ER was discovered and named (Porter *et al.* 1945), and between 1950 and 1970 there were repeated reports of ER viewed with optical microscopy. While some of these publications carefully examined the structure of interest with different microscopical techniques, others based their conclusions on ambiguous data. Interpretations of similar photomicrographs differed widely amongst the various authors. Again, the phase-dark lines in Fig. 6 could be interpreted as filaments or small tubules, or as boundaries between adjacent vacuoles. This period, between 1940 and 1970, is of particular interest, since it contains key publications leading to the present confusion about the identity of the MVS. Although ER mysteriously disappeared from optical microscopes between 1970 and the mid-1980's (other cytological structures came into vogue) it has recently made a reappearance. Just as phase contrast microscopy in the 1960's opened the plant cell to new optical resolution, video enhanced-DIC (VE-DIC) microscopy has uncovered a wealth of previously undescribed structure. The ER is visible, but is quite different from the ER observed with phase contrast in the 1960's. However, the phase contrast observations of the

1960's and the current work with VE-DIC share the same problem. In fact, the situation with DIC is worse, since the vacuolar space is no longer distinguishable from cytoplasmic space, and vacuolar boundaries take on a definite filamentous appearance. Therefore, conclusions are drawn from ambiguous images without any experiments to test hypotheses. This review will examine the post-1940 work with two purposes in mind. Initially, I want to compare the interpretations of different authors who were looking at the same structure. I would then like to propose critical tests these authors might have used to discern the exact nature of their membrane systems of interest.

Fig. 5. Heidenhain's (1907) drawing of a living *Cucurbita* hair viewed with dark field (from Küster, 1935). Fine lines in the cytoplasm probably correspond to boundaries of cytoplasmic vacuoles observed with phase contrast. Magnification is unknown but is presumed to be about 300X.

Fig. 6. A tomato petiolar hair cell viewed with phase-contrast. The vacuoles (one is bracketed by arrows) in this aggregation are separated from each other by fine phase-dark lines. Some chloroplasts (large arrow) can be observed (1900X).

Fig. 7. Hair cell viewed with DIC optics. The boundaries between adjacent vacuoles (one vacuole is bracketed by arrows) now look filamentous, and it is difficult to distinguish between the vacuolar and cytoplasmic space. Once again, vacuolar size can be compared to nearby chloroplasts (large arrow) (2000X).

pre-1940

The variable form of vacuoles attracted attention more than a century ago in connection with the function of carnivorous plants. Darwin (1875) observed that the distribution of a coloured substance within the tentacle cells of the insectivorous plant *Drosera rotundifolia* was altered when the tentacle was stimulated. He observed that a single large coloured body broke up into many smaller ones (a process he termed "aggregation" because the colour became more intense in the smaller bodies). Unfortunately, Darwin incorrectly identified the coloured masses as cytoplasm, an error that was corrected by other researchers at the time, namely Schimper, Gardiner, de Vries (see Lloyd, 1942) and Pfeffer (1887). Eventually, Darwin's son, Francis (Darwin, 1876) concurred that the coloured bodies were, in fact, vacuoles. Åkerman (1917), a visiting scholar in Pfeffer's laboratory, provided important quantitative information about these vacuoles when he investigated their changing osmotic and volumetric properties during aggregation. He also presented the first photomicrographic time-series recording the dynamic organization and complex form of the long cylindrical vacuoles which result from aggregation.

While Åkerman investigated cellular organization using centrifugation and osmotic experiments, there were other novel approaches attempted. Scarth (1927) used micrurgical techniques on various plant cell types to observe cellular responses to physical manipulation. Scarth noted that the cytoplasm in damaged cells vesiculated, but that healthy cytoplasm appeared fibrillar. However, Scarth's observational technique has an important influence on his interpretation of the observed structure. The boundaries between adjacent vacuoles may seem fibrillar when viewed under bright field optics with the condenser diaphragm closed down (a technique Scarth would have used). Owing to the lack of contrast between the vacuoles and cytoplasm, vacuoles are not easily detected against the cytoplasmic background except after

vesiculation. Scarth concluded that cytoplasm was fibrillar, but he could well have been observing an MVS similar to that in the tomato hair cells. Scarth's interpretation is interesting because we will see that it bears close resemblance to later interpretations of living cytoplasm. However, there were others at this time who were working with cells containing more easily detectable vacuoles. Using neutral red as a vital stain, Bailey (1930) demonstrated the seasonal vacuolar transformations in cambial fusiform initials. During the growing season these cells possess a single large vacuole, while in the winter their vacuome consists of a system of long thin vacuoles. Guilliermond (1933), rather than employing vital stains, used cells with vacuoles containing natural pigments (e.g. rose leaf epidermal cells), and reported vacuolar rearrangements during cellular development. He found that as the cells matured, many small, elongated vacuoles fused into a single large, central vacuole. Guilliermond also performed plasmolysis experiments with tulip petal epidermal cells (which also contain conspicuous vacuoles). During plasmolysis a single large vacuole breaks up into many elongated, motile vacuoles, and reassembles when the osmotic influence is removed. Guilliermond compared his results to those of Åkerman (1917) who also showed that the vacuolar transformations in the tentacle cells of *Drosera* leaves were accompanied by changes in cellular osmotic pressure. Therefore, the vacuome was seen as a dynamic and responsive structure, not confined to a single, simple form.

During this period, there evolved various techniques for detecting the Golgi apparatus in plant cells. Guilliermond (1933) was concerned with the artefacts produced by these methods. He found that two common techniques, the silver and the osmic acid methods, yielded very different results. After using these methods on the same cell types, Guilliermond concluded that the two methods were differentially altering vacuoles, permitting two different, but equally erroneous, interpretations of a

single cytological entity. Küster (1935) also expressed his concern about the growing preoccupation with identifying Golgi apparatus. Küster complains (my translation of the German text*):

"There is not much gained, it seems to us, by this comparison [between Golgi and various observed cytoplasmic structures], when so little is dependably known about the Golgi apparatus, and the histochemical behaviour of these plant and animal cell components tells you as little of their organic comparability as their external form does. No wonder, that various authors apparently find entirely different forms in the plant cell similar to the Golgi-apparatus: indeed there are few plant cell components that have not been considered!"

Zirkle (1937) echoed these sentiments by asserting that too little was known about the Golgi apparatus, and it required a more precise definition before homologies between the Golgi and plant vacuoles could be assessed. This conflict over how visual data should be interpreted foreshadowed a similar confusion concerning the identification of

* "Mit diesem Vergleich ist - so scheint uns - schon deswegen nicht viel gewonnen, weil über die Natur des Golgi-Apparatus so wenig Zuverlässiges bekannt ist, und das übereinstimmende histochemische Verhalten dieser und jener Bestandteile der tierischen und pflanzlichen Zelle ebensowenig über deren organische Vergleichbarkeit aussagt wie Übereinstimmungen der äußern Form. Kein Wunder, daß verschiedene Autoren offensichtlich ganz verschiedenartige Gebilde der Pflanzenzelle mit dem Golgi-Apparat vergeichbar finden ... : es gibt wohl nur wenige Bestandteile der Pflanzenzelle, die nicht irgendwann einmal mit ihm gleichgestellt worden wären!"

ER in the optical microscope. Just as Golgi apparatus was novel in the 1930's, and researchers rushed to identify it in any number of cell types, so plant cell microscopists embraced the ER in the 1950's and 60's, and rushed to detect it with their new phase contrast optics.

1940-1970

Endoplasmic reticulum was described by Porter *et al.* (1945), and named by Porter and Kallman in 1952. Initially, Porter's group worked with air-dried whole tissue culture cells, and with this method the membrane system appeared as a reticulum of tubes and vesicles with diameters no more than about 0.1 μm (it is ironic that Porter and Kallman refer to the ER as a "finely divided vacuolar system"). With advances in tissue fixation and microtomy, analysis of very thin sections of tissue became feasible, providing higher resolution (but more difficult to interpret) cross sections of the three dimensional reticulum. Palade (1956) suggested the following criteria for identifying ER in thin sections: a) size of profiles between 0.05 and 0.3 μm in diameter, b) relatively electron transparent content surrounded by a thin dense membrane, and c) an apparent lack of internal structure. Gunning and Steer (1975) provide a good guide to discerning ER in thin sections, and in their plates 21-23 a variety of forms are illustrated. The profiles are all less than 0.1 μm in diameter. In their plate 23c, smooth ER (diameter about 0.05 μm) is contrasted against cytoplasmic vacuoles (diameters 1 μm and greater). Steer and Newcomb (1969) observed very dilated forms of smooth ER in the cells of bean leaf glands, but even the widest tubules were less than 0.05 μm in diameter. Similarly Jørgensen *et al.* (1977) describe dilated cisternal ER but again even the most distended cisternae, which are quite exceptional, are less than 0.8 μm in diameter.

Considering the small size of both tubular and cisternal ER one would not expect to regularly detect the system with optical microscopy. However, Fawcett and Ito (1958) used both phase contrast optics and transmission electron microscopy to view ER in guinea pig testicular cells . They found that the “tubular elements of the endoplasmic reticulum could not be resolved with the phase microscope, but it was possible to see optical sections of the cisternae”. This paper is of extreme importance to subsequent reports of ER viewed with phase contrast optics, and it is worth some space to let Fawcett and Ito give their own account:

“These [fine dark lines] are arranged concentrically and tend to run parallel to the cell surface. Some can be followed for half to two-thirds of the distance around the cell. As the microscope is focused from higher to lower levels these dark contours shift laterally, but their continuity and their relative position is maintained. Therefore, they cannot be interpreted as filaments, but must be regarded as optical sections of extensive sheets or lamellae. Inasmuch as electron micrographs of similar material show pairs of membranes disposed circumferentially in the cytoplasm, the dark lines observed at the periphery of the living spermatocytes are believed to correspond to cisternae of the endoplasmic reticulum”.

Similarly, Porter and Machado (1960) presented some phase contrast and electron micrographs of onion root tip cells. Thick sections (0.5 to 2 μm) of fixed and methacrylate embedded tissue were cut and viewed with phase contrast optics. In these

sections Porter and Machado observed fine phase-dark lines which corresponded to cisternal profiles in electron micrographs. They used these observations to argue, as did Fawcett and Ito with their optical sections, that cross sections of cisternal ER could be observed with phase contrast optics.

There is little doubt that the above publications were indeed reporting cisternal ER viewed at the level of the light microscope. However, those who would later cite this work often made the unwarranted generalization that fine dark lines implied ER. For example, Rose and Pomerat (1960), using phase contrast microscopy, identified ER as fine dark lines in animal tissue culture cells. They did not make any distinction between cisternal and tubular ER. Nor did they discuss the three-dimensional configuration of the fine dark lines, which could easily be determined by adjusting the plane of focus. They also did not provide parallel electron microscopy examining the relative distribution or size of ER at a higher level of resolution. The appearance of fine dark lines seemed proof enough. During the 1960's there was a series of publications describing phase contrast investigations of ER in living plant cells. These publications share similar photomicrographs and data, but differ widely in their interpretations.

Solberg and Bald (1962) identified a system of motile "cytoplasmic vesicles" within the hair cells of *Nicotiana* leaves. The fine dark lines running between the vacuoles were interpreted as intervening cytoplasmic space. At the end of their discussion, however, Solberg and Bald speculate that the fine dark lines may in some way represent ER bounding the individual vacuoles and cite Rose and Pomerat (1960) (and the questionable interpretation therein) as evidence of ER detectable in light microscopy. Similarly, Honda *et al.* (1964) described a "cytoplasmic network" which surrounded small vesicles or vacuoles within tomato petiolar hair cells. Like Solberg and Bald, they felt that the fine dark lines of "the network may be a visible

manifestation of endoplasmic reticulum (ER)". More importantly, Honda *et al.* (1962) produced a film of their observations and presented it at the 1965 Botanical Congress in Edinburgh. Honda *et al.* used this film to illustrate their notions about the cytoplasmic network and ER. However, the people I have spoken with who actually viewed the film were left with a false impression of the film's message. They felt that Honda *et al.* were asserting that the vacuoles framed by the cytoplasmic network were identified as ER. Since this confusion is prevalent and has given me such difficulty, I would like to provide an excerpt from the video based upon the original film.

"The cytoplasmic network is a complicated anastomosing system of vesicles and strands that appear as a continuum. This network appears to be the light microscope equivalent of the endoplasmic reticulum observed in electron micrographs of sectioned cells. The network is always conspicuous in hair cells but not so often in mesophyll cells. The strands of the network are in constant motion and exhibit mitochondria-like movement. Dark strands of the network comprise streaming cytoplasm or kinoplasm. The strands of kinoplasm seem to interlace an invisible and more rigid material, the cortical gel which lines the periphery of the cell. We may think of the cell walls as the walls of a box. The cortical gel then would be an extremely thin, transparent layer of porous sponge lining all the inner surfaces of the box. It is through this porous lining that the kinoplasm and organelles move. The pores of the cortical gel provide a matrix for separation and order in movements of streaming organelles and kinoplasm".

The juxtaposition of images showing cytoplasmic vacuoles with vague speculations about ER, caused the resulting confusion. The popular misconception about this film

and the actual interpretation by the authors has been difficult (or impossible) to dispel. However, there was other work at the time to muddy the waters even further.

Thaine (1961, 1964) also used phase contrast microscopy to describe a membrane system identical in form and behaviour to that reported by Solberg and Bald (1962) and Honda *et al.* (1962, 1964). Thaine had his own interpretation.

"ER tubules, approximately 0.25 μm in diameter, were observed as an integral part of the actively streaming regions of the cytoplasm...The tubules appeared as groups of approximately parallel fine dark lines".

In his drawings (Fig. 3a, 1964), one can clearly see that he interpreted ER tubules as being discontinuous fine dark lines (much like the parallel filaments in Rose and Pomerat, 1960). However, Thaine's identification of this membrane system as ER was overshadowed by the system's proposed involvement in his translocation hypothesis. Because of the scientific community's rejection of his hypothesis, all of Thaine's related work was largely ignored, as often happens when a researcher attacks a ruling paradigm. Thaine's interpretation of his images differs from the interpretations in Solberg and Bald (1962) and Honda *et al.* (1962, 1964) by being less vague about the fine dark lines, and by completely missing the intervening vacuoles. Where the others used words like "manifestation of the ER" or "optical representation of the ER", without being specific about cisternal or tubular ER, Thaine came right out and proposed that the dark lines directly corresponded to ER tubules. Thaine cites Porter and Machado (1960) to support his view, apparently not realizing that the fine dark lines in their work were not tubules, but cross-sections through broad, optically dense cisternae. As to the vacuoles, other workers at this time did see them and included them in completely different interpretations.

Jarosch (1961) described a system of "Bläschenvakuolen" within living cells of *Nitellopsis stelligera* cells. The vacuoles were long tubules or spherical vesicles, and because they were evident in healthy streaming cells he did not believe they were symptomatic of cell damage. He did note, however, that the tubular vacuoles broke into spherical vesicles if the cells were damaged. It should be noted that, at this time, vesiculation was believed by some to be a property associated with the ER. This goes back to the original electron micrographs of the ER in Porter and Kallman (1952). The ER in Porter's early work was highly vesiculated due to excessive extraction during fixation (16 h treatment with osmium tetroxide). Jarosch speculates at the end of his analysis that the vacuoles could be produced from the ER by some sort of surface tension effect. Url (1964) reported a system of "intraplasmatISChe Vakuolen" in onion epidermal cells, and was impressed by their superficial similarity to ER: i.e. their association with the nucleus, and their tendency to vesiculate. The idea of vesiculation as a property of the ER was given added weight by the work of Drawert and Mix (1963). Drawert and Mix observed mitochondrion-sized vesicles (an order of magnitude smaller than Url's vacuoles) attached to the cisternal ER in onion epidermal cells. However, these cells were fixed with potassium permanganate, which is known to effect both extraction and rearrangement of the cytoplasm (O'Brien and McCully, 1981); Url (1964) himself expresses caution about these observations. Even while vaguely comparing the "intraplasmatISChe Vakuolen" to the ER, Url hastens to add that the vacuoles are too large to be ER. However, in 1967 Url apparently decided that the vacuoles must be a dilated version of ER, and he and Bohlar-Nordenkamp (1965), and Bohlar-Nordenkamp (1966, 1968) alone, published a series of papers which make this proposal. It is interesting that Url and Bohlar-Nordenkamp justified their ability to optically detect ER by citing Fawcett and Ito (1958). They completely ignored the

difference between the broad thin cisternal sheets observed in that earlier paper and their description of ER as large phase-bright vacuoles. I do not understand the implied connection between their dilated ER tubules, and the cisternal sheets in Fawcett and Ito (1958). Url and Bohlar-Nordenkampf demonstrated the behaviour of the vacuoles under a variety of conditions (especially vesiculation with mechanical damage, heat, or radiation) but at no time produced electron microscopical, or histochemical evidence to support their claim that the vacuoles were distended ER. The assumption remained an assumption. Feldman (1966) followed the interpretation of Url and Bohlar-Nordenkampf but felt that the long phase-bright vacuoles were ER dilating in response to increasing water uptake by the cell. Feldman believed that the vacuoles were a pathological form of ER resulting from preparation of the onion epidermal peels. However, similar systems can be observed in cells which have been subjected to minimum (or no) preparation (e.g. cells from the alga *Bryopsis*; Menzel and Schliwa, 1986; and the hair cell in Fig. 6).

In contrast to these other publications, Drawert and Rüffer-Bock (1965) described a membrane system in onion epidermal cells very similar to the reticulum originally observed by Porter *et al.* (1945). Unlike the others, Drawert and Rüffer-Bock used a fluorochrome, chlorotetracycline, to visualize the ER. Chlorotetracycline becomes about 500X more fluorescent when it is complexed with Ca^{2+} ions (Reiss and Herth, 1978; Caswell and Hutchison, 1971). Drawert and Rüffer-Bock not only clearly showed a membrane system in onion epidermal cells that was identical to accepted ER structure, they also uncovered the importance of the ER as a cellular Ca^{2+} compartment. Recently, there has been a series of papers using a particular family of fluorochromes (carbocyanines) for visualizing ER in living cells. Inspired by the work of Terasaki *et al.* (1984) with animal tissue culture cells, some plant microscopists have

taken to applying the dye to plant cells (e.g. Quader and Schnepf 1986, Allen and Schumm 1990, Pierson *et al.* 1990). Terasaki *et al.* (1984) did employ parallel staining and electron microscopy to support their identification of the ER. The new dye, 1,3-dihexyloxacarbocyanine (DiOC₆) is a general lipid stain (Terasaki, 1989) but seems to stain ER more intensely than other membrane systems. The image of the ER in onion bulb scale epidermal cells stained with DiOC₆ is the same as when labelled with chlorotetracycline. Obviously, the observations in Drawert and Rüffer-Bock contradicted interpretations of Url and Bohlar-Nordenkamp. Bohlar-Nordenkamp (1968) admitted that such netlike structures in the cytoplasm were difficult to interpret, and suggested that often they represented damaged and vesiculated regions of the cell. However, the large tubules observed by Bohlar-Nordenkamp and Url do not stain with tetracycline (Drawert and Rüffer-Bock, 1965) or DiOC₆. Nonetheless, even modern reviews cite Url's work when discussing ER viewed at the optical level (e.g. Hepler *et al.*, 1990), and make little or no mention of Drawert and Rüffer-Bock.

post-1970

After about 1970, reports of ER visible with phase contrast considerably decreased in frequency. Of course, phase contrast optics were still in use, but DIC was becoming a standard means with which to examine living cells. Unfortunately, workers often use one or the other, although comparing results from both techniques would avoid incorrect interpretations. For example, Ott and Brown (1974) published photomicrographs of "actin microfilaments" in *Vaucheria litoria* viewed with DIC optics. The authors demonstrate that these actin filaments vesiculate when the cell is fixed with glutaraldehyde. The structures they interpret as filaments are really boundaries between long, parallel, closely packed vacuoles, just as Scarth was likely observing. In DIC, the boundaries appear fibrillar, but in phase contrast the phase

differences between the interior of the vacuoles and bulk cytoplasm is discernible. The vesiculation of actin should have alerted the authors to an error in their microfilament theory, especially if they had been familiar with the phase contrast literature of the 1960's (e.g. work by Url and BohlarNordenkamp). In addition, only one year earlier O'Brien *et al.* (1973) had shown that chemical fixation causes vesiculation of an extensive cytoplasmic vacuolar system in petiolar hair cells of *Heracleum mantegazzianum*, an observation which would have cleared up Ott and Brown's confusion over the vesiculation of "microfilaments" during fixation. Similarly, Koop and Kiermayer (1980) reported that "striations" in the cytoplasm of *Acetabularia* became "foamy" when the cells were treated with certain microtubule-disrupting drugs. Like Ott and Brown, these authors used DIC optics and mistakenly identified the boundaries of long cylindrical vacuoles as filaments or striations.

In 1978 Mersey and McCully published work extending the earlier observations of O'Brien *et al.* The emphasis in this work was placed upon the unreliability of conventional fixation techniques in providing a faithful representation of the living cell. In showing that fixation creates dramatic artefacts in the plant cell, they undermined any theories which might have their basis in these same artefacts. However, fixatives have been known to cause artefacts for some time. Mersey and McCully (1978) were the latest to show that fixation alters cytological structure, but certainly not the first. Darwin (1876) described the effects of osmic acid upon the long canalicular vacuoles in *Drosera* tentacle cells:

"...the change was conspicuous and consisted in the transformation of the ropes of aggregation-matter into chains of delicate spheres, reminding one of rows of dewdrops on a spider's web; this change occurred very generally throughout the tentacle."

(Darwin's spider web analogy comes closer to the mark than he might have supposed, see Chapter 2).

Although a modern and more extensive demonstration of fixation artefacts was important, interpretations of the phase contrast investigations of the 1960's confused the issue. In the minds of many, the phase contrast images published by Mersey and McCully (1978) were immediately associated with ER, with little thought to the original data prompting this association. In asserting that the images in Mersey and McCully represented ER, critics often cited for evidence Honda *et al.* (1964) (although these authors were concerned with the fine dark lines between the vacuoles), or Url (e.g. 1967) (although Url in no way ever provided evidence that the vacuoles were ER-associated). The vague speculations of the 1960's had become cemented into conventional wisdom, and this misunderstanding is prevalent to this day. It is interesting that the confusion between vacuoles and ER seems confined only to the cell types used in those important phase-contrast investigations of the 1960's: namely, petiolar hairs (from *Nicotiana* for Honda *et al.*, 1962, 1964) and onion bulb scale epidermal cells (see references for Url and Bohlar-Nordenkamp). The difficulty in distinguishing between the two membrane systems did not arise with other cell types.

For example, Palevitz *et al.* (1981) described a reticular, motile vacuolar system in developing guard cells of *Allium*. Like the vacuoles described by Bailey (1930) and Guilliermond (1933), the vacuoles in the *Allium* guard cells separate and fuse with each other, constantly modifying the system. Later, Palevitz and Hodge (1984) used chlortetracycline to optically reveal ER in developing guard cells of *Vicia faba*, and osmium ferricyanide to stain the ER in *V. faba* and *Allium* for the transmission electron microscope. These two observational techniques allowed comparison between ER and

cytoplasmic vacuoles in the same cells. In the guard cells of both *V. faba* and *Allium*, the ER consists of an array of 0.1 to 0.2 μm diameter tubules connecting flattened cisternae (see Fig. 4 in Palevitz and Hodge, 1984). Individual cytoplasmic vacuoles are separate from the ER, and are 1-10 μm in diameter (see Figs. 2A and 4 in Palevitz and Hodge 1984, and Figs. 8 and 9 in Palevitz *et al.* 1981). Another interesting report of a vacuolar system secondary to a central vacuole came from Menzel and Schliwa (1986) for the siphonous alga *Bryopsis*. The similarity in dimensions, morphology, and motility between the tomato hair MVS and the system in the *Bryopsis* is striking.

Perhaps the most important evidence against Url's interpretation of the cytoplasmic vacuoles comes from the new method to examine ER in living cells (Terasaki *et al.* 1984). As mentioned earlier, this method uses the fluorochrome DiOC₆, which stains mitochondria at lower concentrations (Johnson, 1981), but reveals the ER at higher concentrations. Quader and Schnepf (1986) found that DiOC₆ stained a reticulum and long filamentous tubules in the inner epidermal cells of onion bulb scales. The electron micrographs in Quader *et al.* (1987) make the small ER-tubules (presumably stained by DiOC₆) and the much larger MVS-derived vesicles clearly distinguishable. Likewise, Lichtscheidl and Url (1987), using ultraviolet (UV) microscopy and DiOC₆, described a reticular and filamentous ER in the same cell type. However, unlike Quader and Schnepf, Lichtscheidl and Url also identified 1-4 μm diameter tubules as ER, adding that the large tubes were more easily seen with phase-contrast microscopy and did not stain with DiOC₆. These tubes were none other than the Plasmaschläuche, later labelled ER by Url and Bohlar-Nordenkamp in the mid-1960's. In supporting their conclusions concerning the large membrane tubules, Lichtscheidl and Url cited Bohlar-Nordenkamp (1966) (who supplied no evidence linking the Plasmaschläuche to ER), and Terasaki *et al.* (1984) (who at no time

described structures similar to the Plasmaschluche). However, the large tubes described by Lichtscheidl and Url (and Lichtscheidl and Weiss, 1988) do not fit with the current view of ER in plant cells (Hepler *et al.* 1990; Kachar and Reese 1988; and compare ER and vacuoles in figures published in Hepler, 1981) or animal cells (Terasaki and Jaffe, 1991; Terasaki *et al.*, 1986 and 1984). Despite data showing that the large 1-4 μm tubules are physically and chemically dissimilar from conventional ER, Lichtscheidl and Url stuck with Url's original interpretation from the 1960's, an interpretation which remains unquestioned even in recent reviews (Hepler *et al.* 1990). However, I believe that as the current DiOC₆-inspired view of ER takes a firmer hold on the literature, the support for large 1-4 μm diameter ER tubules will diminish. Presently, more concern should exist over the tendency of modern plant cell biologists to view living cells with only DIC optics (the problems inherent in this approach were illustrated with Ott and Brown, 1974). Rather, a broader experimental approach should be taken so that conclusions will not rest on equivocal data. For example, Terasaki and Jaffe (1991) are cautious about their ability to use the fluorochrome DiIC₁₈ (a lipophilic dye with a longer alkyl chain than DiOC₆) to visualize the ER in sea urchin eggs, and use immunohistochemical data (Henson *et al.*, 1989) and calcium localization (Terasaki and Sardet, 1991) to support their interpretation of ER morphology. However, simpler tests than these can help in interpreting membrane structures in living plant cells.

For example, Pierson *et al.* (1990) combined DIC microscopy with DiOC₆ staining and UV microscopy to identify motile ER tubules in pollen tubes of Lilium longiflorum. Pierson *et al.* observed that fine (0.3 μm diameter) filaments were visible with DIC and UV microscopy, and similar filaments stained with DiOC₆. The resemblance between the structures revealed by these various techniques is striking. Although Pierson *et al.* did use a range of observational techniques, it would have been

useful to have observed the appearance of the DIC- and DiOC₆-visualized filaments after fixation. If the fine lines were actually boundaries of a vacuole then the vesiculation pattern would be distinctive. Otherwise, if the fine filaments are indeed ER then they should be stable with conventional fixation techniques (Terasaki *et al.* 1984, and Figs. 24-27).

Wilson *et al.* (1990) demonstrated that membrane cylinders followed predictable patterns when breaking up into spherical vesicles. These patterns are expected to occur independently of cylinder size. For example, if the phase-dark lines in Fig. 6 represent individual tubules, then their disintegration would result in vesicles with a radius of about 0.1 μm . The vesiculation of the small phase-dark tubules would not influence the intervening phase-bright regions. Alternatively, if fixation causes small phase-dark tubules to self-organize into circular units (which is one interpretation for the vesiculation image, see Figs. 11-13), then in DIC these tori will appear as filament rings (Allen and Brown, 1988 interpret vesicles as filament rings). Electron micrographs should occasionally reveal sections through these small tori. This is not observed. If the phase-bright regions are long, cylindrical vacuoles with phase dark boundaries, then fixation will produce spherical vesicles about 1 μm and larger. Electron micrographs show that the fixed cytoplasm contains many cytoplasmic vesicles having the same radius as those observed in phase contrast and DIC. Therefore, observing the fixation of cells allows one to test hypotheses concerning the actual three dimensional form represented in a two-dimensional photomicrograph. Other critical tests using vital stains and osmotica can be applied, and these will be covered in Chapter 3.

Science is popularly believed to progress by the steady accretion of fact upon fact, publication upon publication, each advance standing on the shoulders of its

predecessor. Any person who critically examines the literature discovers that the above description of Science's stately advance is sometimes untrue. Scientific literature reflects and reports a human endeavour, and therefore contains some error and redundancy. When the system is operating ideally, the accumulation of many facts may lead to a realignment of thinking (a paradigm shift), or the completion of a standard list (e.g. the periodic table, or a taxonomy). However, when the information base is used carelessly or improperly, the literature may become strewn with misdirection and confusion. Signposts are knocked down; trails are trampled. I have certainly detected such misdirection and confusion in literature describing ER and vacuolar systems. Good solid pre-1940's work is ignored in favour of fashionable speculation in the 1960's. I believe that plant cell microscopy should be more experimental, rather than descriptive. It is much more difficult to misquote hard data than it is to misinterpret vague propositions. Chapters 2 and 3 carry on from this experimental philosophy by quantitatively examining the vesiculation event, and discovering properties of the MVS which suggest critical tests for distinguishing vacuoles from ER. I believe this ability to distinguish between different membrane systems in a living plant cell has posed problems in the past, and should become more important to plant cell biologists as improved imaging techniques cause microscopists to identify systems not previously detectable at the optical level.

Chapter 2. Modelling vesiculation

Introduction

The vesiculation of the MVS during cytological fixation is more than just the response of an isolated cellular system to changing conditions. Instead, the pattern of vesiculation will be influenced by the response of the entire cell to the fixative. However, the whole-cell response might only be detectable through such cytological indicators as the MVS. Assuming this, it is perhaps best to investigate how the initial conditions of the vacuoles determines vesiculation patterns. For example, the extent and type of disruption of the MVS during fixation depends very much upon the fixative used. Coagulant fixatives (e.g. formalin-acetic acid-alcohol) dismantle and eventually dissolve the MVS (Mersey, 1977). In contrast, non-coagulant fixatives (e.g. formaldehyde, glutaraldehyde) vesiculate the MVS: that is, the vacuoles transform from cylinders to spheres. One of the first effects of a non-coagulant fixative is the cessation of cytoplasmic streaming. Then, depending on the fixative, there may be a thinning and folding of the MVS after streaming has stopped. This thinning and folding is most noticeable with acrolein (O'Brien and McCully, 1981; p. 4.15) or a modified Karnovsky fixative (2% paraformaldehyde-3.2% glutaraldehyde) (Mersey and McCully, 1978). The vacuoles of the MVS can vesiculate during this folding process, but those which do so earlier produce only a few vesicles with large diameter, while those vesiculating later produce many vesicles of smaller diameter (see Figs. 11 - 13). Although vesiculation has been reported in previous work, there has been little attempt to quantitatively analyse the details of this membrane transformation. The

patterns observed during this process may provide important clues for a predictive explanation of vesiculation, and of the kinds of alteration that should be allowed for when extrapolating from dead specimens to live cells.

The complex patterns described above might inspire a variety of complicated hypotheses about vesiculation (for example, transformations of surface proteins, etc.). However, simple systems constrained by only a few rules may generate complex patterns and behaviours. Occam's razor stipulates that a simple approach be applied before resorting to more complicated hypotheses. The most obvious place to begin is by envisioning the geometric consequences of a cylinder (an individual MVS vacuole) transforming to several spheres (vesicles). The mathematical model described below begins with this premise, and throughout the modelling process the model is compared to reality and adjusted if theory diverges from observation. These validations of the model provoke a consideration of osmotic conditions during fixation, and the permeability properties of the MVS membrane. Understanding the behaviour of the MVS during fixation ultimately leads to interesting insights into basic properties of semipermeable membrane cylinders and the fundamental importance of surface area to volume relationships.

Although the mathematical model is intended to use geometric considerations to explore the vesiculation process, this approach does not give us information about the physico-chemical causes of vacuolar breakdown. One way to investigate the physical effects of fixatives on membranes is to use artificial membranes as a model for the MVS. Leathes (1925) showed that myelin forms (cylindrical growths which arise from hydrated lecithin) provide a good system for testing the effects of different chemicals on membranes. He observed interesting differences in the development of the myelin forms when lecithin was hydrated with solutions of different salts. Of particular

interest was calcium, which produced effects quite different from other cations, both mono- and divalent. While myelin forms developed in solutions containing the other cations, they did not arise in the calcium solutions (the cations shared the same counterion). However, the other cations did influence the shape of the myelin forms, causing a greater frequency of spherical forms. A study similar to Leathes' will be performed here, but instead of observing the effects of different chemicals on myelin form development, I will treat fully developed myelin forms with chemicals and observe changes in morphology. If fixatives are directly causing vesiculation, then a comparable phenomenon should be observed with myelin forms exposed to glutaraldehyde. However, vesiculation may be caused indirectly by fixation through changes in the cell's ionic or osmotic environment. These conditions will be simulated by treating myelin forms with: 1) different salts (NaCl, CaCl₂, and MgCl₂) to test the effects of various ions, and 2) sucrose solutions to discover the effects of changing osmotic conditions on the morphology of myelin forms.

In the previous chapter I discussed the work of Drawert and Rufferbock (1967) who used tetracycline to visualize Ca²⁺ compartments in onion epidermal cells. Subsequently, the Ca²⁺ compartment revealed by Drawert and Rufferbock was found to be ER. Since chlortetracycline represents a well-known probe for calcium, and no similar probes exist for potassium and magnesium ions, I will use this stain to investigate changing calcium distributions in onion epidermal cells during fixation. The onion cells are better for this procedure due to the relative impermeability of the hairs' radial cell walls. Like Drawert and Rufferbock, I will use chlortetracycline to locate the ER-compartmentalized Ca²⁺, but will also determine whether fixation has an effect on this pattern of Ca²⁺ localization.

Materials and Methods

A) The Mathematical Model

Assumptions of the model

In creating a mathematical model, important properties of the system are abstracted and incorporated into a mathematical analogy. The behaviour of the mathematical system is then compared to that of the observed system. The similarity between simulation and reality reveals whether the elements chosen adequately describe the system. Adopting simplifying assumptions is an important step in reducing a natural system to what appear to be its rudiments. I made the following assumptions: 1) membrane area is constant, 2) vesiculation of a cylinder is instantaneous and volume is not lost during or after vesiculation, 3) the osmotic influence of the fixative solution is constant over time, and 4) the MVS membrane is impermeable to solutes. Each of these assumptions will be examined in turn.

1. Constant membrane surface area

In order to establish that membrane area was conserved during vesiculation, I found photomicrographs in Mersey (1977) in which it was possible to follow isolated vacuoles through vesiculation. I found that the number of vesicles which result from a given vacuole is consistent with an assumption of conserved membrane area (this represents a preliminary test of equation 2). Therefore, an assumption of constant surface area is reasonable for further modelling MVS vesiculation.

2. Instantaneous vesiculation

The model is simplified if it is assumed that the transition from cylinders to spheres is instantaneous. In reality, one may observe a vacuole vesiculating over a few seconds. Such observations reveal that vesiculation of these vacuoles is an example of "necking" (the breakdown of fluid cylinders investigated by J.A.F. Plateau in the late nineteenth century, see Plateau, pp. 370-373 in Thompson 1942). The intermediate forms between cylinder and spheres are solids of revolution called unduloids. Their geometry is not simple and would be very difficult to model. Since the time taken to vesiculate (a few seconds) is small compared to the time the vacuoles are exposed to fixative (a few minutes), I assume that there is no volume lost during a virtually instantaneous vesiculation.

3. Constant osmotic influences

There is some dispute over the osmotic effects of fixatives, indeed there is a question whether fixatives exert any osmotic influence at all. There is some evidence supporting the hypothesis that fixatives are effectively osmotically inactive (eg. Young, 1935; Arbough *et al.*, 1976), and evidence contradicting this (Coetzee and van der Merwe, 1985, Taylor, 1988). Some of the controversy arises from the fact that these studies use a variety of tissue types: suspended rat liver cells (Arbough *et al.* 1976), fish scales (Bone and Denton, 1971), perfused rat lung (Mathieu *et al.*, 1978), *Nitella flexilis* internodal cells (Taylor, 1988) and marine invertebrates (Young, 1935). Taylor's evidence is particularly persuasive. He found that the loss of turgor caused by exposure to fixatives was nearly equivalent to the osmotic activity of the fixative as measured with vapour pressure or freezing point osmometry (see table 1 in Taylor, 1988). After being exposed to fixatives for about an hour, the *Nitella* cells lost their

ability to hold turgor. I will show here that the vacuoles of the MVS also behave as osmometers in the presence of fixatives.

4. Permeability of the MVS membrane to fixatives

In the absence of any data on the reflection coefficient of the MVS membrane for various osmotica, a reflection coefficient of unity (maximum reflection of solute and hence maximum fixative osmotic potential) is assumed for the vacuolar membrane. Since vesiculation occurs within a few minutes, I will also assume that the permeability of the MVS membrane to fixatives does not change and that the fixatives exert a constant osmotic influence.

Table 1. An alphabetical list of the symbols (with their magnitudes or units) used for mathematical modelling of MVS vesiculation.

Symbol	Definition	Units
A	total membrane area (time invariant)	m^2
l	length of a cylindrical tube	m
l_0	tube length at time zero	m
l_t	tube length at time (t)	m
L_w	hydraulic conductivity of membrane	$m\ Pa^{-1}\ s^{-1}$
n	number of spherical vesicles	-
n	osmolarity of solution	(milliosmoles)
r	radius of a spherical vesicle	m
R	radius of a cylindrical tube	m
R_0	tube radius at time zero	m
R_t	tube radius at time (t)	m
$\mathcal{R}$	gas constant	$8.3143\ m^3\ Pa\ mol^{-1}\ K^{-1}$
Δt	time from time zero to time (t)	s
T	temperature	K
V	tube volume = vesicle volume	m^3
V_0	tube volume at time zero	m^3
V_t	tube volume at time (t)	m^3
ΔV	volume change in tube	m^3
V	volume	m^3
Ψ_0	osmotic potential	MPa

Conversion of cylinders to spheres

Constant volume

The modelling procedure begins with examining the vesiculation event geometrically. Consider each initial intact vacuole in the MVS as a right circular cylinder (the ends of the cylinders are assumed to have negligible area, see Fig. 8). Vesiculation is a transition from this simple cylinder to a number (n) of spheres, all of radius r (see Fig. 8). If the area of the cylindrical vacuole is equated with the total vesicle area (assumption 1), and likewise the vacuolar and vesicular volume are made equal (assumption 2), then

$$2\pi Rl = n4\pi r^2 \quad \text{and} \quad \pi R^2 l = n \frac{4}{3} \pi r^3 .$$

It follows that $n = \frac{R}{2r^2} l$ and $n = \frac{3R^2}{4r^3} l$.

In consequence, $r = \frac{3}{2} R$ (1)

and $n = \frac{2l}{9R}$ (2).

The relationships in (1) and (2) were compared with vesiculation events recorded in Mersey (1977). Whenever equation (1) was found to hold, then (2) was also true. However, the ratio between r and R was often less than $\frac{3}{2}$, which I believed indicated a loss of vacuolar volume. The model needed to be adjusted to account for systems where equations (1) and (2) do not apply.

Changing volume

Assumption 2 (constant volume) applies only to the transition from cylinder to spheres. However, a cylinder may change volume, thus acquiring new dimensions, before it vesiculates. It is assumed that, once formed, a vesicle does not change in volume. This can be observed directly in Mersey and McCully (1978) where vesicles do not change in size over time. Consider a cylinder, with an initial radius of R_0 and length of l_0 , which decreases in volume to reach a new radius (R_t) and length (l_t) (Fig. 9). The area (A) is described by

$$A = 2\pi R_0 l_0 = 2\pi R_t l_t \quad (3),$$

the initial volume (V_0) by $V_0 = \pi R_0^2 l_0$,

and the final volume (V_t) by $V_t = \pi R_t^2 l_t \quad (4).$

From (3)
$$l_t = \frac{R_o l_o}{R_t} \quad (5),$$

and
$$l_o = \frac{A}{2\pi R_o} \quad (6)$$

Substituting (5) and (6) into (4) yields

$$R_t = \frac{2V_t}{A} \quad (7).$$

So R_t must be used in equations (1) and (2) for vesiculations occurring after time zero.

Fig. 8. Representation of a cylinder transformed to a system of spheres where surface area and volume are conserved.

Fig. 9 The transition from cylinder to spheres illustrated on the left occurs with conservation of area and volume while the transition on the right occurs after the cylinder has lost one half of its volume but retained its original area.

Osmotic effects, water fluxes

The cylindrical vacuoles may decrease in volume (lose water) because of osmotic effects of the fixative. Since area (A) is assumed constant, it follows from equation (7) that if volume decreases, then so does radius (R_t), while the length at any time (l_t) increases. These osmotic influences may be described using an equation for the flux of water across a membrane (Davson and Danielli, 1970):

$$\Delta V = L_w \Psi_o \Delta t A \quad (8).$$

Equations (5) and (7) predict that the vacuoles of the MVS become longer and thinner as they lose volume. The final cylinder radius can be expressed in terms of the initial cylinder radius:

$$R_t = \frac{2V_t}{A} = \frac{2(\Delta V + V_o)}{A} = \frac{2(V_o + L_w \Psi_o \Delta t A)}{A} \quad (9),$$

and with vesiculation $r = \frac{3}{2} R_t = \frac{3(V_o + L_w \Psi_o \Delta t A)}{A}$

which simplifies to $r = \frac{3}{2} R_o + 3L_w \Psi_o \Delta t \quad (10).$

Equation (10) predicts that as osmotic influences increase, the radii of the vesicles will decrease (because Ψ_o will be negative). Like equation (1), equation (2) is based upon the radius and length of the tube which vesiculates, not the radius and length of the initial tube. We may incorporate the osmotic effects of the fixative into equation (2) in the following way:

from equation (5) and substituting (7) into (2), $n = \frac{l_t A}{9V_t}$

from which
$$n = \frac{R_o l_o}{2r^2} \quad (11).$$

Equation 11 rewritten as its reciprocal is

$$\frac{1}{n} = \frac{9(V_o + L_w \Psi_o \Delta t A)}{l_t A}.$$

$$= \frac{9}{2} \frac{R_o}{l_t} + \frac{9L_w \Psi_o \Delta t}{l_t}$$

$$= \frac{3r}{l_o} \left(1 + \frac{\Delta V}{V_o} \right) \quad (12).$$

Equations (11) and (12) predict that as osmotic effects increase, the number of vesicles will increase (because ΔV will be negative). We can now test the model by calculating the number of vesicles that arise from a cylindrical vacuole in any given osmotic environment. Predicted values for n will be compared to actual values found in photomicrographs in Mersey (1977). However, to proceed further we need a value for the hydraulic conductivity (L_w) of the MVS membrane.

Hydraulic conductivity of the MVS membrane:

An expression for hydraulic conductivity is obtained by rearranging equation (8):

$$\begin{aligned}
 L_w &= \frac{\Delta V}{\Psi_o \Delta t A} \\
 &= \frac{(\pi R_t^2 l_t - \pi R_o^2 l_o)}{2 \Psi_o \Delta t 2 \pi R_o l_o} \\
 &= \frac{R_t^2 l_t}{2 \Psi_o \Delta t R_o l_o} - \frac{R_o^2 l_o}{2 \Psi_o \Delta t R_o l_o}
 \end{aligned}$$

Equation (5) can be written as

$$\frac{l_t}{l_o} = \frac{R_o}{R_t}$$

$$\text{so } L_w = \frac{R_t^2 R_o}{2\Psi_o \Delta t R_o R_t} - \frac{R_o^2 l_o}{2\Psi_o \Delta t R_o l_o} = \frac{R_t - R_o}{2\Psi_o \Delta t} \quad (13).$$

Therefore, L_w can be calculated by following the change in vacuolar radius caused by a known osmotic influence. I measured the radii (R_o and R_t) of individual vacuoles in hair cells exposed to three different osmotica: 1) a modified Karnovsky fixative (2% paraformaldehyde-3.2% glutaraldehyde in 0.05M HEPES with 0.1M CaCl_2 and 0.005M MgCl_2) (these measurements were taken from Figs. 9, 10, and 12 in Mersey and McCully, 1978 and are copied here as Figs. 10-12 for reference), 2) 0.15 M sucrose (Figs. 13 and 14), and 3) 0.13 M DMSO (Fig. 15). This last osmoticum was chosen because later experiments with the actin-disrupting drug cytochalasin D (described in Chapter 3) indicated that the solution was producing osmotic effects. Since the drug was in micromolar concentrations (2-20 μM), the solvent (0.13 M DMSO) was assumed to be the important osmoticum. Ten individual values of L_w from each treatment (30 values in all) were used to determine a mean hydraulic conductivity. Osmotic potentials of the various osmotica were calculated using osmolarity measurements and the van't Hoff relation:

$$\Psi_o = \frac{nRT}{V}.$$

Osmolarity was measured on a freezing point osmometer (Osmette S, Precision Systems, Inc.).

Figs. 10-12. Correspond to Figures 9, 10 and 12 in Mersey and McCully (1978). Effects of a modified Karnovsky fixative (2% paraformaldehyde-3.2% glutaraldehyde) in 0.05M HEPES buffer containing 0.1M CaCl_2 and 0.005M MgCl_2 on a tomato petiolar hair cell. A chloroplast (C) provides a fixed point around which the vacuoles (arrows) of the MVS undergo thinning and folding (Fig. 11) and eventual vesiculation (Fig. 12). Fig. 10 shows the hair cell 1 min after addition of fixative, Fig. 11 at 4 min and Fig. 12 at 10 min (3400X).

Figs. 13 and 14. A hair cell treated with 0.15 M sucrose and viewed with phase-contrast. The radius of the vacuoles (arrows) has decreased by one third in the 4.5 min which has elapsed between Fig. 13 and Fig. 14 (2640X).

Fig. 15. A hair cell treated with 0.13 M DMSO (with 10 μM cytochalasin D) and viewed with phase-contrast. The vacuoles of the MVS (one vacuole is bracketed by arrows) have decreased in radius and folded due to changing osmotic conditions (1930X).

The computer simulation

The computer program was coded in standard PASCAL and compiled on the Carleton University CP-6. The algorithm for the computer model is given in Fig. 16. The program uses two arrays, the first contains the information for a system of cylindrical vacuoles (R_0 , l_0 , A , and V_0), while the second records the information for the spherical vesicles which arise from each vacuole. The vesicle array lists vesicle number and radius, the area and volume for each vesicle, as well as the dimensions of a residual oblate spheroid (an ellipsoid). This residual ellipsoid results when the calculation for vesicle number turns up a fractional sphere. Rather than just truncating the vesicle number to an integer, the fractional value is used to generate an ellipsoid. Two of the axes of the ellipsoid are made equal to the radius of the other vesicles, while the third axis is the radius of the spherical vesicle multiplied by the fractional part of n . When the simulation is initiated the first (vacuole) array contains information for ten tubes and the second (vesicle) array is empty.

The vacuole array is initialized by inputting mean values for initial vacuole radius and length. With this information the program generates ten values of radii and lengths that are normally distributed around the means provided (with a standard deviation 0.1 of the mean). The program then calculates area and volume for each vacuole. One of these vacuoles is randomly selected for vesiculation. Values for vesicle radius and number are calculated using equations (1) and (2) and loaded into the vesicle array. The fractional portion of n is used to calculate the axes, area and volume of the residual ellipsoid.

The program then simulates the osmotic effect of a fixative. If the operator inputs mean values for hydraulic conductivity and osmotic potential, then the program

generates values for these two variables that are distributed normally around the given means (standard deviation 0.1 of the mean). The program uses these values to calculate the volume decrease for each of the vacuoles. After the osmotic effects have been calculated, the program vesiculates another vacuole, and repeats the procedure until no vacuoles remain. The output is recorded in a separate file for subsequent analysis.

The computer simulation was used to compare the behaviour of the mathematical model with vesiculation of the real vacuoles in Figs. 10-12. Mean vesicle radius for Fig. 12 was determined by drawing four transects across the photomicrograph and sampling vesicles along those transects. Mean vesicle radius was then calculated for the results of five computer simulations. Initial vacuole radius, and hydraulic conductivity were matched to the conditions in Figs. 10-12 ($1.25\ \mu\text{m}$ and $10^{-15}\ \text{m Pa}^{-1}\ \text{s}^{-1}$, respectively), and only the simulated fixative osmotic potential was varied. The simulation with a value for osmotic potential closest to the measured value for the Karnovsky's fixative is expected to produce a mean vesicle radius most similar to that measured for Fig. 12.

Fig. 16. The algorithm describing the steps followed by the computer model simulating the effects of fixation on the MVS.

B) The Physical Model

Myelin forms

Using a sharpened wooden stick, a small amount of lecithin (L- α -phosphatidylcholine, Sigma Chemical Co.) was smeared onto a clean glass microscope slide. A glass cover slip was then placed over the dry lecithin smear. The dry smear was viewed with phase contrast optics while distilled water was drawn under the coverslip. In this way, the formation of myelin forms could be observed. Once the movement of the myelin forms ceased, experimental solutions were drawn under the coverslip. These experimental solutions were:

- 1) 3% glutaraldehyde,
- 2) 50 mM CaCl_2 ,
- 3) 50 mM MgCl_2 ,
- 4) 50 mM KCl, and
- 5) 150 mM sucrose.

The effects of these different solutions were observed with phase contrast optics and recorded in photomicrographs.

Chlortetracycline

Pieces of onion bulb scale epidermis (see page 79 for the preparation of these epidermis pieces) were floated on solutions of 0.01% (aqueous) chlortetracycline hydrochloride (Sigma Chemical Co., Lot no. 78F0022) for 15 - 30 min. Staining was

observed with epifluorescence optics. The effects of fixation were examined by fixing epidermal cells before or after staining with chlortetracycline. Fixation was by floating pieces of tissue in 2.5% glutaraldehyde (buffered in 0.025 M dibasic potassium phosphate, pH 6.8) for 3-5 min. Controls were run by excluding the fixative, the buffer, or the stain.

Photomicrography

Phase contrast microscopy was with an Olympus Vanox microscope with 20X SPLAN and 100X oil immersion SPLAN objectives. Chlortetracycline-treated samples were viewed with an Olympus CH-2 microscope with blue exciter filter BP-490 and beam splitter DM-500+O-515. Images were recorded on Kodak T-MAX 100 and 400 black and white film and developed in Kodak T-MAX developer.

Results

A) The Mathematical Model

Predictions of vesicle numbers

Expected values for vesicle numbers (Table 2) were calculated according to equation (11). An observed number of vesicles (Table 2) was derived by first calculating final vacuolar length (l_t),

using equation (1) substituted into equation (5)
$$l_t = \frac{3R_0l_0}{2r} , \quad (14)$$

and then counting vesicles along this length.

Table 2. The calculated and observed number of vesicles from MVS vesiculations illustrated in Mersey (1977)

Figs.	expected n	observed n
30-31	6.7	8
31-32	10.5	9
26-27	7.6	7
27-28	10.7	11

The agreement between observed and expected is very close ($\chi^2 = 0.522$, 3 degrees of freedom, $P = 0.914$), supporting the validity of the model.

Hydraulic conductivity of the MVS membrane

A sample calculation and the mean value for L_w are given below:

osmolarity = 522 milliosmoles, 1.184 MPa

$R_0 = 0.5 \mu\text{m}$

$R_t = 0.2 \mu\text{m}$

$t = 180 \text{ s}$

$$L_w = \frac{(R_0 - R_t)}{2\Psi_0\Delta t} = 0.79 \times 10^{-15} \text{ m Pa}^{-1} \text{ s}^{-1}$$

There was some variation in the values for R_0 and R_t within one series of photomicrographs. This range in the values for the initial and final radii produced the

variance in the final means for L_w calculated from each osmotic treatment (Table 3). Thirty separate calculations (ten from each osmoticum) produced a mean L_w of $(1.06 \pm 0.21) \times 10^{-15} \text{ m Pa}^{-1} \text{ s}^{-1}$.

Table 3. Mean values of hydraulic conductivity (L_w) for the MVS membrane in hair cells using a sample size of ten for three different osmotica.

Osmoticum	Osmotic potential (MPa)	L_w ($\text{mPa}^{-1}\text{s}^{-1}$)
Karnovsky fixative*	1.184	$(0.68 \pm 0.21) \times 10^{-15}$
DMSO	0.252	$(0.79 \pm 0.32) \times 10^{-15}$
sucrose	0.363	$(1.70 \pm 0.51) \times 10^{-15}$

*Data taken from Mersey and McCully (1978).

NB. The larger value of L_w for sucrose is discussed below.

Computer simulations

The patterns of vesiculation modelled in computer simulations resembled actual vesiculation. Vacuoles that vesiculated earlier produced the fewest and largest vesicles, but vacuoles which vesiculated later produced progressively both more and smaller vesicles (Fig. 17). Additionally, the computer simulation with a fixative osmotic potential closest to the measured value resulted in a mean vesicle radius most similar to the actual value (Table 4) (see raw data for simulation 4 in Appendix 1). It is also interesting that the standard deviation relative to the mean increases with increasing fixative osmotic potential.

Table 4. Simulated values for mean vesicle radius* under varying simulated osmotic conditions compared to the observed mean vesicle radius in Fig. 13.

system	fixative osmotic potential (MPa)	mean vesicle radius (μm)
Fig. 13	1.184	0.53 ± 0.20
simulation 1	0.35	1.81 ± 0.90
simulation 2	0.50	1.70 ± 0.86
simulation 3	0.80	0.88 ± 0.69
simulation 4	1.0	0.45 ± 0.37
simulation 5	1.8	0.20 ± 0.23

***Values for simulated mean vesicle radius are calculated from twenty iterations.**

Fig. 17. A sample of a simulation where $R_0=1.25\ \mu\text{m}$, $l_0=15\ \mu\text{m}$, $\Psi_0=0.8\ \text{MPa}$, and $L_w=10^{-15}\ \text{mPa}\cdot\text{s}^{-1}$. Time sampled: a) 0 min., b) 5 min. and c) 10 min. The cylinders become longer and thinner with increasing time before vesiculation and cylinders which vericulate last produce many small vesicles.

B) The Physical Model

Myelin forms

When the lecithin becomes hydrated long cylindrical myelin forms grow out from the smear and are moved across the field of view by water rushing under the coverslip (Fig. 18). The myelin forms come in a range of diameters (about 1.4 to 14 μm) and are not simple cylinders. There may be loops at one end, or a thick myelin form may attenuate sharply into a much thinner cylinder.

Glutaraldehyde has no effect on the shape of the artificial membranes (Fig. 19). In contrast, all of the salts caused the myelin forms to either break up into vesicles, or condense into thin fibrils (Figs. 20-22). Sucrose does not cause vesiculation or condensation, but instead produces helical coiling in some of the myelin forms (Fig. 23).

Chlortetracycline

Treatment of onion epidermis with chlortetracycline labels certain organelles within the epidermal cells (Figs. 24 and 25). Staining occurs in mitochondria and in a fine cortical reticulum (Fig. 24). The fine lines making up the reticulum connect with broad chlortetracycline-positive sheets (Fig. 24). These sheets can be found surrounding the nucleus (Fig. 25). The nucleus itself appears unstained. Fixation dramatically alters the pattern of staining whether the fixative is applied before or after the chlortetracycline (Fig. 27). Fluorescence disappears from the cortical reticulum and the mitochondria. Buffer does not alter normal staining patterns (Fig. 26). Coarse strands and clumps of cytoplasm become stained after fixation (Figs. 26 and 28), but do not appear in unstained, fixed tissue (Fig. 29). The nuclear fluorescence observed

after fixation (Fig. 26) seems fixative-induced, since it appears in fixed tissue which is unstained (Fig. 29).

Figs. 18-23. Fig. 18 shows an untreated myelin form which has developed from hydrated L- α -phosphatidylcholine. These membrane cylinders come in a range of diameters (1040X). Addition of 3% glutaraldehyde in Fig. 19 (750X) has no effect on the morphology of the myelin forms. However, Figs. 20-22 show that cations (50 mM Ca²⁺, Mg²⁺ and K⁺, respectively) disrupt the myelin forms, creating a high frequency of vesicular shapes. Osmotic influences (Fig. 23) do not cause the same effect. Rather, treatment with 150 mM sucrose caused helical coiling in many of the myelin forms (Fig. 20, 1300X; Figs. 21 and 22, 1040X; Fig. 23, 1144X).

Figs. 24 and 25. Onion epidermal cells stained with 0.01% chlortetracycline for 20 min.

The fluorochrome has labelled the mitochondria (m), and a reticulum (connecting elements of reticulum are bracketed with small white arrows) in the cortical cytoplasm. This reticulum contains broad chlortetracycline-positive sheets (s). The nucleus (N) (which is unstained) is always connected to some of these sheets (Fig. 24, 750X; Fig. 25, 580X).

Fig. 26. Pre-treatment with buffer (0.025 M dibasic potassium phosphate, pH 6.8) does not alter the pattern of staining. The cortical reticulum is still visible (connecting elements bracketed by arrows, sheets indicated by 's'), as are long chlortetracycline-positive filaments (F) running through streaming cytoplasm just interior to the cortical cytoplasm (594X).

Fig. 27. Cells in the same epidermis as above after treatment with 2.5 % glutaraldehyde (in 0.025 M dibasic potassium phosphate buffer, pH 6.8). Staining of the reticulum has disappeared. The nucleus (N) and coarse cytoplasmic strands (c) have become fluorescent (580X).

Fig. 28. After fixation, clumps of cytoplasm (C) (which surround dark vesicles, v) become chlortetracycline-positive (660X).

Fig. 29. The nuclear fluorescence (N) observed in Figs. 27 and 28 also occurs in unstained, fixed tissue (495X).

Discussion

A) The Mathematical Model

Beginning with simple assumptions about vacuole shape, the mathematical model shows that the thinning and folding of the vacuoles during fixation are caused by the osmotic influence of the fixative. As mentioned in the introduction to this chapter, acrolein seems to cause the greatest degree of thinning and folding (Mersey, 1977), which may be explained by the relative abilities of the different fixatives to penetrate the radial walls of the hair cell. Glutaraldehyde can enter only by the base of the hair cell, via the subtending cells, thus giving a distinctive fixation front as it diffuses along the cell (Mersey and McCully, 1978). The slower rate of penetration with glutaraldehyde means that it is present at lower initial concentrations, giving time for fixative to diffuse into the MVS. In contrast, acrolein freely diffuses across the radial wall and enters the cell at high initial concentrations. Osmotic effects will be more pronounced, resulting in changes in vacuolar morphology, which in turn will affect the final vesicle size and number. Once the osmotic effects were appreciated, I was able to predict the size and number of vesicles resulting from a given vacuole (for example, vesicle numbers in Table 2). Although there were only a few time series in Mersey (1977) which allowed this sort of careful analysis, the examples in Table 2 demonstrate that the initial assumptions for the model are reasonable. Having shown that the model could predict fixation patterns, it was not necessary to repeat work already performed by Mersey (1977).

Treatment with the three osmotica has shown that the fine lines visible in phase contrast or DIC microscopy actually represent boundaries of membrane bound spaces. If an osmoticum acted from outside of the cell (as was true for sucrose) with water

leaving the cytoplasm we would expect to see the fine lines come closer together, even if they did represent separate filaments. However, osmotica acting from within the cytoplasm (for example, the fixative solution) would not cause the fine lines to pull together unless the lines represented the boundary of a space enclosed by a semi-permeable membrane. This observed pattern represents a test which could be used to distinguish between vacuoles and ER-tubules in a living cell. The Karnovsky fixative (Figs. 10-12) and the DMSO (Fig. 15), being within the cytoplasm, exerted their osmotic effect directly upon the vacuoles of the MVS. It is doubtful that the reflection coefficients for these two solutes is unity (i.e. the MVS membrane is completely solute impermeable), as is assumed, and so we would expect that the theoretical osmotic potential difference is not realized (because solute can leak in). The calculated hydraulic conductivity would therefore be lower than the actual value (see equation 1 in Materials and Methods). Sucrose (Figs. 13 and 14), however, is not expected to freely cross the plasmalemma, and its osmotic effects would be communicated via the intervening cytoplasm. This would represent a situation where the solute's reflection coefficient is effectively unity. The actual osmotic potential difference approaches the theoretical value, and the calculated hydraulic conductivity will be higher. This pattern is reflected in the results (Table 2) where the value for the Karnovsky fixative and DMSO are lower than that calculated with sucrose.

The model, while predicting changes in vacuole morphology, assumes that there are no osmotically driven changes in vesicle size and shape. I made this assumption because, in all of the published photomicrographs showing MVS fixation, I could detect no evidence of vesicles being affected in this way. For example, in Fig. 11 in Mersey and McCully (1978) there are vesicles which continue to have the same size and shape when observed in their Fig. 12, even while adjacent vacuoles thin and

elongate. I do not know why this should be, but I can propose three possible explanations for why vesicles might be osmotically unaffected by fixative.

1. Changes in shape may not be easily detected in vesicles due to their close physical association. Therefore their morphological stability may be an observational artefact.

This explanation would not apply so well to large, easy to measure vesicles which maintain their size and shape even while neighbouring cylindrical vacuoles lose volume.

2. Previous work (Taylor, 1988) has indicated that biological membranes become more permeable to solutes (e.g. buffer ions, fixatives) after exposure to fixatives. This change in permeability might explain the geometric stability of the vesicles.

For example, Taylor found that *N. flexilis* cells recovered about 84% of their turgor after treatment with 1% glutaraldehyde (this recovery only came after an initial turgor loss of about 50%). Subsequent treatments with glutaraldehyde caused the cells to lose turgor completely at about the 60 min mark. At this time turgor dropped suddenly over a time interval of about 10 min. Therefore, it seemed that about an hour was required for the fixative to have its destructive effect on the membrane, while the water flux coincident with turgor loss required only a few minutes once the damage was done. Similar treatments with formaldehyde resulted in turgor loss at about the 150 min mark. Again, once turgor loss was initiated, pressure drop required only 10-20 min to be complete. Since vesiculation requires only a few minutes, aldehyde fixatives would not be expected to greatly affect the semi-permeability of the MVS membrane within the time frame of vesiculation. Therefore, the insensitivity of the vesicles to the same

osmotic forces felt by their "parent" vacuole is puzzling. The transition from vacuole to vesicle seems to incur more than just a change in morphology, and the physical state of the vesicles needs to be more closely examined.

3. The vesicles would be in an unusual state from the time of vesiculation to when changes in semi-permeability are assumed to occur. During this time the vesicle experiences a reverse osmotic pressure, which tends to collapse rather than expand the surface. These surface forces are best described by LaPlace's equation (Thompson, 1942), which relates surface tensions with pressure and curvature. There is also the inward directed pressure generated by the surface tension of the membrane itself, which increases when the membrane becomes spherical (Thompson, 1942). Both of these negative pressures are balanced by the positive pressure of the contained fluid. The spherical shape of the vesicles presents a stable minimum surface area to this balance of pressures.

I favour this third explanation. The state of osmotic tension which may exist in the vesicles could be simulated mathematically, or with a physical model (e.g. artificial vesicles).

It was possible to test the mathematical model on a large number of simulated vacuoles using computer programming. The simulated fixative osmotic potential which produced a mean vesicle radius similar to the observed value falls between 0.8 and 1.0 MPa, and the measured fixative osmotic potential for the fixation shown in Figs. 11-13 is 1.184. Therefore, the model seems to underestimate fixative osmotic potential. This underestimation might be for the reason discussed below relating to effects of fixatives on the semi-permeability of biological membranes. However, the simulated value for hydraulic conductivity has sufficient error and approximation to make more complicated

explanations for the model's underestimation unnecessary. More importantly, the model came reasonably close, and predicts that mean vesicle radius becomes more uniform (lower relative variance) as fixative osmotic potential decreases.

Particularly important in the osmotic properties of the vacuoles is their initial radius. This dependence upon radius is understandable when one appreciates the relationship between relative volume loss (percent volume lost per unit time) and the osmotic environment. Relative volume loss is given by

$$\frac{\Delta V}{V} \frac{1}{\Delta t} = \frac{L_w \Psi_o \Delta t A}{V} = \frac{2L_w \Psi_o}{R_o} \quad (17),$$

or more simply and in differential terms

$$\frac{dV}{dt} \frac{1}{V} \propto \frac{1}{R_o} \quad (18).$$

The rate of change of relative volume is inversely proportional to the radius of curvature (equation 18) making a smaller vacuole a more sensitive osmometer than a larger vacuole. A smaller vacuole has a greater surface area to volume ratio so it will lose more relative volume per unit time. A good analogy might involve a mouse and an elephant. A mouse will lose a greater percentage of its body heat on a cold morning than an elephant because the mouse has a greater surface area to volume ratio. Some plant cells (e.g. guard cells, pulvinus motor cells, and *Drosera* tentacle cells) have been observed to alter the morphology of their central vacuole from many smaller vacuoles to

perhaps just one large vacuole (Couot-Gastelier *et al.*, 1984; Campbell and Garber, 1980; Campbell and Thompson, 1977; Satter *et al.*, 1970; Åkerman, 1917). This change in vacuolar area to volume ratio would alter the function of the vacuole as an osmotic "balloon". Another important advantage of a greater surface area to volume ratio is that smaller vacuoles may fuse into fewer, larger vacuoles to accommodate expansion of vacuolar volume.

The hydraulic conductivity of the MVS membrane ($1.1 \times 10^{-15} \text{ mPa}^{-1}\text{s}^{-1}$) is less than would be expected by most plant physiologists. More acceptable would be 10^{-12} or $10^{-13} \text{ mPa}^{-1}\text{s}^{-1}$, values found for the plasmalemma of *Chara* cells and leaf mesophyll cells, respectively (Nobel, 1983). This may be explained by assuming that single cells adapt to their particular surface area to volume ratio (i. e. their tendency to lose or gain water osmotically) by altering their hydraulic conductivity. For example, Nobel (1983) calculates that the L_w for a *Chara* cell, with radius of about 500 μm , is of the order of $10^{-12} \text{ m Pa}^{-1} \text{ s}^{-1}$. If cells adjust their membranes for greater surface area to volume ratios by decreasing membrane permeability, then the L_w of the MVS membrane would be about three orders of magnitude less than that of the algal cell plasmalemma. This would make the L_w for the MVS membrane about $10^{-15} \text{ m Pa}^{-1} \text{ s}^{-1}$, which is the same order of magnitude as our measured L_w . The plasmalemma of sieve tubes, with a radius one order of magnitude greater than the MVS, would have a permeability coefficient of about $10^{-14} \text{ m Pa}^{-1} \text{ s}^{-1}$ (one measured value is about $2 \times 10^{-14} \text{ m Pa}^{-1} \text{ s}^{-1}$: Weatherly *et al.*, 1959).

This consideration of the hydraulic conductivities of various membrane cylinders brings out an important point concerning the surface area to volume ratio of the large central vacuole found in many plant cells; for example, the central vacuole in the tomato hair cells. This central vacuole occupies most of the cell volume and has

acquired a relatively constant surface area to volume ratio. Any drastic change in this ratio would require the central vacuole to fragment into many smaller vacuoles. This is not observed. Therefore, if the hair cell is under water stress (as such a cell must commonly be), then water will leave the cytoplasm and be replaced by vacuolar water. However, the tonoplast of the central vacuole has a set surface area and hydraulic conductivity. It is capable of only a certain maximum hydraulic flux. If water stress places a demand on cytoplasmic water such that water loss exceeds the maximum available hydraulic flux across the tonoplast, then metabolic function will be endangered. This danger might be removed by a secondary vacuolar system with a greater surface area to volume ratio than the central vacuole. The MVS not only provides an increase in available surface area for water exchange, but is also actively distributed through the cell such that local water demands might be constantly supplied. The changes in the radius of MVS vacuoles during plasmolysis with sucrose indicates that these vacuoles are supplying water to surrounding cytoplasm. Therefore, the MVS might serve in a transport function, circulating bulk water through the cell and providing a large surface area for water flux. It is interesting that hair cells always possess a more extensive MVS than cells which do not experience the same degree of water stress (e.g. onion epidermal cells, Wilson *et al.* 1992, in preparation).

The importance of the surface area to volume ratio (first recognized by Archimedes) cannot be overstated. An appreciation of this relationship makes other properties of the MVS less puzzling. For example, the vacuoles of the MVS within a given cell have a fairly regular radius (see Fig. 11). Now one can see that since the vacuoles are composed of similar membranes with similar permeability, they have tended towards a similar radius (area to volume ratio) in a common physical

environment. It is interesting that the ER, which is about one order of magnitude less in radius, would clearly possess a membrane with very different properties.

Morphological changes, like the thinning and lengthening of membrane cylinders, are sure clues to the internal osmotic environment of a cell. This consideration should be especially important to workers observing osmotic effects on whole cells, or fine morphological features in chemically fixed cells. For example, Bray *et al.* (1991) examined the importance of positive turgor pressure in the growth of cultured chick neurons. Estimations with a vapour pressure osmometer and direct measurements with a pressure probe suggested an internal hydrostatic pressure differential of about 0.01 MPa (0.1 atm). The authors assumed that by increasing the osmolality of the growth medium, the positive internal pressure would disappear, causing arrested filopodia growth. The cultured neurons were exposed to media with an osmotic shift of +20, +50, and +100 mosM (equivalent to osmotic potentials of -0.05, -0.13, and -0.25 MPa, respectively). Bray *et al.* observed that in these hypertonic solutions, the filopodia increased in length within 3-5 min., rather than showing the expected slowed growth. In fact, the filopodia double in length when exposed to the 100 mosM treatment. This is roughly predicted by equation (17). The filopodia have a radius of about 0.3 μm (as indicated by the photomicrographs). However, cell radius is on the order of a couple micrometers, so the hydraulic conductivity of the neuron plasmalemma should be about $10^{-15} \text{ m Pa}^{-1} \text{ s}^{-1}$, (see above for the proposed relationship between surface area to volume ratios and hydraulic conductivity). The rate of relative volume change for the filopodia can be calculated (where R_0 in equation 17 will equal filopodia radius):

$$\begin{aligned}\frac{dV}{dt} \frac{1}{V} &= \frac{2 (10^{-15} \text{ m Pa}^{-1} \text{ s}^{-1})(0.25 \times 10^6 \text{ Pa})}{0.3 \times 10^{-6} \text{ m}} \\ &= 0.2 \% \text{ per second} \\ &= \text{about } 50\% \text{ in } 4 \text{ min.}\end{aligned}$$

Since the relative change in length is the inverse of relative volume change (see Fig. 9), the filopodia are expected to roughly double in length in this time period under these osmotic conditions. However, the authors concluded that elongation of filopodia could not be turgor-driven. Bray *et al.* made the mistake of comparing the behaviour of naked membrane cylinders with that of fungal hyphae, which cannot uniformly elongate under osmotic forces because of cell wall constraints.

An example for the importance of recognizing changes in area to volume relationships can also be found for intracellular structure. Gonzalez-Reyes *et al.* (1989) have recently shown that Golgi cisternae have smaller diameters with increasing fixative osmolarity. This result is predicted by the model if we assume, as Gonzalez-Reyes *et al.* did, that a single cisterna is a disc (i.e. a cylinder whose radius is much greater than its length). The model predicts that as fixative osmolarity increases, the diameter of each cisterna will decrease and the cylinder length will increase. Along these lines, suppose a researcher observes that the Golgi apparatus changes when cells go from one state to another, for example, healthy to diseased (we will assume that the fixation procedures are identical in all of the treatments used by the researcher). A change in morphology (e.g. a smaller diameter and wider lumen) may not be due to different properties of the Golgi. Instead, the morphological differences might be caused by the cells having a different osmotic potential in their two states. This example illustrates how a single observation may be interpreted in different ways. It also illustrates why cell microscopists must always be conscious of the physical consequences of their

methodologies, and how individual organelles do not respond as isolated systems to external influences but reflect the changing state of the entire cell.

Although the breakdown of myelin forms in the presence of different ions will be discussed in a following section, I believe the helical coiling of the myelin forms under osmotic influences fits more appropriately at this juncture, since it involves the osmotic response of membrane cylinders. Myelin forms actually consist of several layers of membranes, but for analytical purposes, we will assume that they are made up of many coaxial membrane cylinders which are physically connected (they are actually membranes rolled up like a jellyroll). Equation (17) predicts that the inner cylinders will lose relative volume at a greater rate than the outer cylinders. That is, the inner cylinders will increase in length more quickly than the outer ones. I believe that it is this differential growth of coaxial cylinders that creates the helical coiling. Interior cylinders elongating at greater relative rates are constrained by the larger radius cylinders immediately exterior to them. This sets up increasing tensile stresses as you move towards the periphery of the myelin form. The helical coiling originates because compressive and tensile stresses always give rise to shears at 45° (Gordon, 1978). This shear in the plane of the membranes sets up a torsion, or twist, in the myelin form which produces the coiling. The principles behind the helical coiling of myelin forms under osmotic influences might have some application to plant growth. The root or shoot is composed of physically continuous coaxial cylinders which experience different tensile stresses (Sachs, 1875). This tension would set up the same torsional forces we have seen in the myelin forms (would this torsional stress align cellulose microfibrils at a 45° angle to the long axis?). I think a study intended to mathematically predict twisting in internodes might lead to some interesting insights into the relationships between plant biomechanics and development.

This mathematical study began as an investigation into the necessary geometric constraints involved in transforming cylinders to spheres. When delving into the shapes of natural forms, there comes an appreciation of the ground rules that all structures must abide by: rules like the surface area to volume relationship. These fundamental ground rules can be scaled up or down to include biological forms of any size. Such scaling considerations should cause cytologists to look with wary eyes upon images of subcellular structures. How much are the observed ultrastructure and dimensions reminiscent of actual function, and how much are they a geometric realization of altered cytoplasmic conditions? This is an old and largely ignored question. The model presented here has shown that an examination of simple geometric forms under the influence of various forces may answer questions about the same forms at different levels of magnitude.

B) The Physical Model

The disruption of the MVS during cell death (induced by either fixation or cell damage) must be an indicator of general physical and chemical alterations occurring throughout the cell. The mechanism of the vesiculation event is unknown but the transitional membrane forms resemble images from Thompson (1942). Thompson described work by J.A.F. Plateau who, in the late nineteenth century, studied the stability of fluid cylinders in terms of their dimensions and their surface tension. Plateau found that a fluid cylinder, past a critical length, would acquire an unduloid

geometry and break into spheres. The critical length could be increased if the surface tension was decreased or if either the viscosity or inertia of the fluid was increased. The experiments with the lecithin model system have shown that these cylindrical membrane forms vesiculate in the presence of cations (Ca^{2+} , Mg^{2+} , and K^{+}) but not fixatives. This is not surprising since vesiculation is observed in cells where fixatives are absent, for example when cells are mechanically damaged. McIver and Schurch (1983) and Schürch *et al.* (1981) showed that the interfacial tension of a phospholipid monolayer increased with increasing Ca^{2+} concentrations. I would therefore hypothesize that both cell death and fixation result in the disruption of Ca^{2+} or K^{+} compartments resulting in increased free ion concentrations. Trump *et al.* (1981) show that there are increases in Ca^{2+} concentrations in damaged or dying cells, and they believe that these increases play a pivotal role in the irreversible processes leading to cell death. Furthermore, Trump *et al.* (1981) state that vesiculation and other membrane rearrangements are commonly observed during cell death. Sjöstrand (1989) comments that cylindrical membranes are particularly vulnerable to vesiculation, which is the pattern one would predict from Plateau's work. I feel that increases in interfacial tensions in cellular membranes, due to a changing cellular environment, could explain this general vesiculation phenomenon. We now see why Darwin (1876) was so close to the mark when he compared the vesiculation of *Drosera* tentacle cell vacuoles to beads of dew on a spider web. Water cannot wet a spider's web as a continuous fluid cylinder (Thompson, 1942). Past a certain length to radius ratio, the cylinder of water goes through the transformation described by Plateau, and breaks up into droplets; just as an elongated vacuole breaks up into vesicles as membrane surface tensions increase. The two phenomena share a physical cause. One has to be impressed by Darwin's uncanny insights.

There are different cellular compartments where ions might leak from after cell damage or fixation. The vacuole might be a source of Ca^{2+} and K^+ , but the time required for the tonoplast to become permeable to such ions during aldehyde fixation is probably in the order of hours (Taylor, 1988). The fixative might deactivate Ca^{2+} -complexing proteins, such as calmodulin, causing an increase in free cellular Ca^{2+} . The domains of the calmodulin molecule which bind Ca^{2+} ions are rich in α -helices (Carafoli, 1987); glutaraldehyde disrupts the α -helical regions of proteins by over 20% within 2 hours, and osmium tetroxide denatures α -helices by over 50% within 30 minutes (Lenard and Singer, 1968). The time frame for the disruption of protein α -helical domains would not seem to fit the time required for vesiculation. Another Ca^{2+} compartment is the ER (Carafoli, 1987; Terasaki and Sardet, 1991), which seemed to lose calcium during fixation (as demonstrated by the loss of chlortetracycline-staining). Before fixation, the chlortetracycline-stained reticulum is identical in organization to that revealed by DiOC_6 , a fluorochrome used to visualize ER in animal and plant cells (see Figs. 36 and 38). The chlortetracycline-stained sheets surrounding the nucleus also stain with DiOC_6 , and have been found to vary in size and organization with intracellular Ca^{2+} concentrations (Quader, 1990). I propose that the loss of chlortetracycline staining after a few minutes of fixation is due to calcium leakage, and that the ER could be a source of free Ca^{2+} ions which contribute to a disruption in the cellular ionic environment during cell death, thus causing changes in membrane conformation. The autofluorescence caused by glutaraldehyde polymerization (Fig. 28), in contrast to the fixative-induced autofluorescence of the nucleus (Fig. 29), may be the result of this post-fixation Ca^{2+} ion release. This cytoplasmic staining appears only where cytoplasm is clumped, and is not as intense as the ER-labelling. This might be due to a lower concentration of calcium ions (the released ions are dispersed through

a greater volume). However, quantitative results (for example, with fura-2; Carafoli, 1987) are required before fixation-induced calcium fluxes can be fully understood.

It is interesting that Ca^{2+} release during fertilization is linked to a dramatic reorganization of the ER in sea urchin eggs (Terasaki and Jaffe, 1991). Terasaki and Jaffe observed that tubular ER was completely disrupted and cisternal sheets became more finely divided during fertilization. These changes were spatially and temporally coincident with a calcium wave progressing through the cell. Apparently unknown to Terasaki and Jaffe, Quader and Fast (1990) observed similar reorganization of ER when acidifying the cytoplasm of onion epidermal cells. Since Felle (1988) has shown that cytoplasmic acidification is accompanied by increases in free Ca^{2+} concentrations, it is reasonable to assume that these separate observations of ER transformations may be linked by a common physical cause, increases in cytoplasmic Ca^{2+} levels. Neither of these publications addresses the phenomenon at the level of material cause. Instead, the authors speculate about the possible functions such ER reorganizations might have. I propose that the disruption of the L.T. tubules, like MVS vesiculation, is simply a response of fluid cylinders to a changing physicochemical environment. The relative stability of the cortical ER during a fixation procedure which vesiculates MVS (see Figs. 39-42) may result from either different properties of the surrounding nonmotile cortical cytoplasm, or contents of the ER possessing a greater viscosity than the contents of the MVS (see Plateau in Thompson 1942 concerning the relationship between the stability of fluid cylinders and their viscosity). If the MVS contains a solution of simple, inorganic ions, then the viscosity of these contents would be close to pure water (about 10^{-2} poise). The proteinaceous contents of the ER could have a viscosity about the same as the surrounding cytoplasmic gel, which is 100-1000 times more viscous than water (Kamitsubo et al., 1988). However, the ER is not exempt

from vesiculation. For example, Porter and Kallman (1952) observed highly vesiculated ER in whole, air dried tissue culture cells. The cells in question in Porter and Kallman showed evidence of considerable extraction owing to extended exposure to osmium tetroxide vapour (Bowers and Maser, 1988). Bahr (1955) found that during a 4 h fixation with 1% aqueous osmium tetroxide over 50% of the proteins can be extracted from a range of animal cells. The vesiculated ER in Porter and Kallman (1952) was observed in cells fixed in osmium tetroxide vapour for 16 h while bathed in a saline solution. The extraction of material important to the viscosity of intra- or extra-ER fluids may have resulted in the vesiculation of the ER. In contrast, a 4 h fixation with 3% glutaraldehyde (pH 7.2 at room temperature) extracts only 10% of proteins from the cells of bean leaves (Coetzee and van der Merwe, 1984). This may leave the viscosity of the ER contents less affected allowing the cylinders of the ER to be preserved.

Regardless of the mechanism of vesiculation, the final images in electron micrographs give no indication of the former dynamic vacuolar system. There are only empty vesicles for which many functions could be imagined. The ascription of functions to artefacts is not without precedent. For example, standard bacteriology textbooks instruct that mesosomes, invaginations of the cell membrane, are regularly found in Gram-positive bacteria. The proposed functions for mesosomes ranged from involvement in DNA replication to energy production. However, Ebersold *et al.* (1981) and later Dubochet *et al.* (1983) showed with cryotechniques that mesosomes were actually artefacts produced by conventional fixation for transmission electron microscopy. Similarly, McCully and Canny (1985) preserved unvesiculated MVS using rapid freezing and freeze-substitution techniques. Likewise, Tiwari and Polito

(1988) observed long, cylindrical vacuoles in rapidly frozen and freeze-substituted pollen tubes of *Pyrus communis*.

Although there is a danger of crystal formation during freezing, if cooling is rapid enough then ice crystals are smaller and will cause no visible damage. The cooling rate will depend to some extent on the thickness of the specimen. For example, a cooling rate of 10^9 K s^{-1} can be attained at a depth of $0.1 \text{ }\mu\text{m}$, but a rate no greater than 10 K s^{-1} will occur at a depth of 1 mm (Moor, 1987). A sufficiently rapid cooling rate will immobilize cellular structure for freeze-substitution. During freeze-substitution, an organic solvent is substituted for water in the specimen (maintained at about -80° C). A fixative (e.g. acrolein) is also introduced during the freeze-substitution process, but since structure is immobilized at -80° C , the fixative cannot cause MVS vesiculation. Plant cells preserved in this manner would be expected to have fewer artefactual vesicles than is found in conventionally fixed tissue; vesicles that would otherwise be assumed to perform functions in the live cell. For example, Hyde *et al.* (1991) have used rapid freezing and freeze-substitution to show that an alternative model must be developed for sporangial cleavage in *Phytophthora*. It had been previously thought that the alignment of cleavage vesicles determined future subdivisions of the sporangium. However, Hyde *et al.* showed with cryotechniques that the cleavage vesicles were actually artefacts of chemical fixation. The vesicles arise from the breakdown of membranous sheets that extend along future subdivision boundaries. Similarly, Heath *et al.* (1985) found that while the wall vesicles in the hyphae of *Saprolegnia* were circular and arranged in linear arrays after chemical fixation, freeze-substitution preserved the vesicles as very elongated cylinders with electron-opaque contents not observed after chemical fixation. I believe that many proposed structures and models will require re-examination as the artefacts of chemical

fixation become better known. It will be interesting to try to use the nature of the artefact to give clues about the properties of the living structure.

Chapter 3. Properties of the MVS.

Introduction

In chapter 1, I discussed how the ambiguity of phase contrast and DIC images can lead to multiple and mutually inconsistent interpretations of the same membrane system. The mathematical model in chapter 2 made reliable predictions of vesicle size and number only because the model was based on a correct interpretation of MVS morphology; vesiculation of the MVS occurs predictably and according to geometric constraints set by initial vacuolar dimensions. While the model has led to a fuller understanding of the vacuolar system, many properties of the intact system remain uninvestigated. This is because the MVS has largely escaped the attention of plant cell biologists due to their current tendency to use only DIC optics to view living cells. The problem inherent in using only one optical technique has been discussed in Chapter 1. I propose that investigating the reaction of different vital dyes with the MVS will not only provide critical tests to distinguish the vacuoles from ER, but will also give information about some properties of this relatively unexplored membrane system. Further properties of the MVS, specifically MVS-cytoplasmic associations also need to be investigated in order to develop some picture of how this vacuolar system fits into the general organization of the plant cell.

Vital stains are used to observe different cytoplasmic or vacuolar components without actually killing cells or seriously disrupting cell function. Since cells are functioning while the staining pattern is observed, some chemical properties of the MVS and its relationship to other organelles (particularly the ER) can be discovered

while the vacuoles are intact. No stain has been shown to label the MVS (Mersey, 1977), but the reactions of various vital dyes may help to indicate ways to distinguish the MVS from surrounding cytoplasm. Three vital dyes will be used for this purpose here: neutral red, a stain commonly observed to accumulate within vacuoles (Stadelmann and Kinzel, 1972); uranin (disodium fluorescein), an anionic fluorochrome which, much like acid fuchsin (Gurr, 1965), binds to cationic substrates (e.g. proteins); and DiOC₆, a general lipophilic stain used to distinguish ER in both animal and plant cells (Terasaki, 1989). Neutral red is thought to accumulate within vacuoles through two primary mechanisms. These consist of either an ion-trap or a chemical reaction process. Neutral red has a pK of 7.38 (i.e. one-half of the dye molecules are uncharged at this pH); therefore, if maintained at the appropriate neutral pH in a buffer, some of the dye diffuses across the plasma membrane and into the cell. Dye molecules then diffuse across the tonoplast and into the vacuole. The ion-trap mechanism requires an acidic vacuolar space which ionizes the majority of the dye molecules (e.g. only 1 in 20,000 molecules will be uncharged at pH 3.38) making most of the dye unable to cross the tonoplast. Alternatively the vacuole may contain tannin-like compounds which will bind the dye molecules through a chemical reaction. The nature of the neutral red reaction (should there be any reaction) will give information about the contents of the MVS (Stadelmann and Kinzel, 1972). In contrast, uranin will be used to stain the cytoplasm surrounding the MVS. As mentioned in chapter 1, much of the confusion concerning the interpretation of DIC images resides in the inability of the microscopist to distinguish between cytoplasmic and vacuolar space. Uranin is shown to be particularly useful in drawing this distinction. DiOC₆ labels neither cytoplasm nor the contents of vacuoles, but instead has been useful in distinguishing ER from the surrounding cytoplasm. Although originally used for the vital staining of mitochondria, at higher concentrations DiOC₆ appears also to stain the ER (Terasaki *et*

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al., 1984). DiOC₆ is not thought to be ER-specific, but labels all membranes (Terasaki, 1989); its ability to reveal fine ER tubules is more a matter of dye concentration than any ER-specificity.

A distinguishing feature of the MVS is its widespread distribution within the cell, a pattern made possible by the system's motility (see Figs. 37-42). The association of vacuoles with their supporting cytoplasm may be profitably investigated through the centrifugation of living cells. David Mottier (1899) (one of Wilhelm Pfeffer's students) was the first to centrifuge living cells and observe their subsequent behaviour. Mottier found that, given time, the contents of many different cell types (for example, *Chara* and *Nitella*) will recover their pre-centrifugation orientation even after extreme centrifugation treatments. This same phenomenon has been observed subsequently in different cell types by many other workers (e.g. Beams and King, 1938; Beams, 1943; Bouck, 1963; Kamitsubo *et al.*, 1989). Tomato hair cells are particularly suited to this technique because their relatively large size makes them easy to orient. I will use centrifugation to discover 1) the relative density of the hair cell contents, 2) how the MVS is associated with the bulk cytoplasm, and 3) the rate of MVS recovery relative to other organelles.

The motility and distribution of a wide range of organelles has been shown to depend on both actin (Sheetz and Spudich, 1983; Lloyd, 1988) and microtubules (Vale *et al.*, 1985). One way to determine which of these cytoskeletal elements is responsible for vacuolar movement is by treating hair cells with actin-disrupting and microtubule-disrupting drugs and observing their relative effects. Mersey (1977) used treatments with colchicine (to disrupt microtubules) and cytochalasin B (for actin) and discovered that vacuolar motility is actin-dependent. I felt that this earlier work might be complemented by comparing the effects of drugs different from those used by Mersey.

To determine the influence of microtubule-disrupting drugs on MVS organization and motility, I, like Mersey, used colchicine, but also nocodazole (Terasaki *et al.* 1986), a drug not tested by Mersey (1977). Cytochalasin D, which is chemically very similar to cytochalasin B, was used to disrupt F-actin (Yahara *et al.*, 1982). Since actin has already been shown to be important in vacuolar motility, I investigated actin organization using a fluorescent label, rhodamine-conjugated phalloidin. Phalloidin is an actin-binding protein from the poisonous fungus *Amanita phalloides* (Merck Index). The rhodamine molecule attached to the phalloidin allows one to detect the actin-phalloidin complex (Parthasarathy *et al.*, 1985). Actin in hair cells will be examined with particular interest in changes in organization after centrifugation and during subsequent cellular recovery.

Materials and Methods

Plant Material

Tomato plants (*Lycopersicon esculentum* Mill.) were grown in a greenhouse to the age of 8-12 weeks. Petiolar hairs were removed without damaging the large basal cells (Mersey and McCully, 1978) and floated on distilled water until needed (some were used immediately while others were left for over 1h). Cooking-onions (*Allium cepa* L.), 5-8 cm in diameter, were bought at local markets and sliced into wedges. The inner epidermis of the bulbscales was cut into 1 cm² sections while still attached then gently peeled off with fine forceps. These pieces floated on distilled water for at least 1h before use. Speed of vacuolar movement in the hair and onion epidermal cells was measured by recording time lapse photomicrophotographs with phase contrast optics.

Vital dyes

Neutral red (Chroma-Gesellschaft, colour index # 50040) was dissolved to 0.1% w/v in 0.025 M potassium phosphate buffer at pH 7.8. Tomato hairs or sections of onion epidermis were immersed in the dye solution and examined with bright field optics at approximately 10 min intervals for up to 30 min. Some hair cells and epidermal pieces were floated in uranin or DiOC₆ solutions (5 to 15 min), mounted in water, and observed with epifluorescence optics. Uranin (di-sodium fluorescein, CI#45350, Chroma-Gesellschaft, Lot No. 1B315) was used at 0.001% w/v in distilled water. DiOC₆ (3-3' dihexyloxacarbocyanine iodide, Sigma, Lot No. 127F3682) was dissolved in absolute ethanol for a stock solution of 0.1% (Terasaki *et al.* 1984). Final working solutions of DiOC₆ were 0.001‰ - 0.005‰ in distilled water. Controls for

all of these stains consisted of cells observed without the dye but in the relevant solvent (for example, 0.1% ethanol for DiOC₆). Some onion cells stained with DiOC₆ were subsequently fixed with 2.5% glutaraldehyde to study the relative effects of the fixative on ER and the MVS. Control cells were treated with the same fixative solution.

Centrifugation

In order to keep the longitudinal axis of the hair cells parallel to the direction of the centrifugal force, a water-filled piece of 1/8 in tygon tubing plugged with a piece of toothpick was used to sheath hairs while they were centrifuged in Eppendorf tubes (Fig. 30). Two centrifugation treatments were used to test the effects of centrifugal forces on subsequent recovery: a weak treatment (4100 g for 7 min) and a strong treatment (8400 g for 14 min). Seven hairs were subjected to each of the two centrifugation treatments. Centrifuged and uncentrifuged hairs were mounted in distilled water for immediate observation with phase contrast optics or stained for actin. Cells recovering from the centrifugation treatments were observed over a time interval of about 3h and the position of cellular components was recorded with phase contrast photomicrographs taken at different times during the recovery process.

Fig. 30. Materials used to maintain the stable position of petiolar hairs relative to centrifugal force. The hairs (h) were kept upright in a plastic tube (t) and held in by a piece of toothpick (p). This assembly was put in water in an Eppendorf centrifuge tube.

Actin labelling

Actin was labelled in both centrifuged and uncentrifuged hair cells using the method in Sonobe and Shibaoka (1989). This actin labelling technique uses *m*-maleimidobenzoyl-*N*-hydroxy succinimide ester (MBS) (Sigma) to stabilize F-actin prior to fixation with formaldehyde. I departed from their method by using 0.025 M potassium phosphate buffer (pH 6.8) rather than phosphate buffered saline (pH 7.0). Following fixation the distal endwall of the hair's basal cell was cut off using iridectomy scissors. This was done to aid the entry of rhodamine conjugated phalloidin (Sigma Chemical Co. St. Louis MO) into the thick-walled cells. Hairs were immersed in buffered rhodamine-phalloidin (0.1 μ M) for 15-30 min and quickly rinsed (about 30 s) in buffer before viewing with epifluorescence optics.

Cytoskeleton disrupting drugs

Cytochalasin D (Sigma) was kept as a stock solution of 2 mM in DMSO (dimethylsulphoxide) (Schliwa, 1982) and diluted in distilled water for working solutions of 2 - 20 μ M. Colchicine (Sigma) was used in aqueous solutions of 12.5 - 125 mM and stored in the dark. A stock solution of nocodazole (methyl (5-[2-thienylcarbonyl]-1H-benzimidazol-2-yl)-carbamate) (Aldrich Chemical Co.Inc., Milwaukee WI) of 1.7 mM in DMSO was diluted in distilled water to produce the working solutions of 3.3 - 33 μ M. The control solutions were 0.13 M DMSO for cytochalasin D and nocodazole, and distilled water for colchicine. Tomato petiolar hairs and onion epidermal sections were mounted in experimental and control solutions and observed with phase contrast optics. Cytochalasin D treatments were monitored

continually until effects were noted. Longer term colchicine and nocodazole treated cells were floated on experimental and control solutions then mounted and examined at 15 min intervals. In order to determine the influence of these changes on cellular recovery following centrifugation, actin and microtubule disrupting drugs (at the above concentrations) were each applied to cells immediately before(1-2 min) and during centrifugation, or immediately after (between 1-2 min) centrifugation. Cells were subsequently observed and compared to controls.

Microscopy and photomicrography

Two optical systems were used. 1) A Carl Zeiss Universal microscope equipped with phase contrast optics and a Zeiss Ukatron UN-60 flash was used for some of the phase contrast work. 2) An Olympus CH-2 was used for some phase contrast and all epifluorescence. Uranin and DiOC₆ treated samples were viewed with blue exciter filter BP-490 and beam splitter DM-500+O-515, and cells stained with rhodamine-phalloidin were viewed with a green exciter filter IF-545 and barrier filter O-590.. Photomicrographs were recorded on Kodak Panatomic X, Plus X, Kodak TMAX 400, and Kodak Technical Pan.

Note about sample size:

No exact figure can be given for the sample size in the preceding staining and centrifugation procedures. However, repeated observations over the course of 2-3 years would put a conservative estimate for sample size for each procedure at more than 25. Qualitative observations reported here are gleaned from numerous, repeatable results.

Results

1) Vital Dyes

a) Neutral red

Neutral red is accumulated by the large central vacuole in onion epidermal cells but not by the MVS. Vesiculation, which occurs in the unhealthy epidermal cells (Mersey, 1977), does not alter the distribution of the neutral red (Fig. 31). The hair cells do not accumulate neutral red in either the large central vacuole (which occupies most of the cellular volume) or the vacuoles of the MVS.

b) Uranin

Uranin acts as a general cytoplasmic stain in both onion epidermal and tomato hair cells (Fig. 32). Cells remain healthy in the dye solution while observed with bright field or phase-contrast, but exposure to the high energy excitation beam results in vesiculation of the vacuoles within a few seconds. Vesiculation caused by viewing cells with epifluorescence meant that photomicrographs had to be recorded before damage was too extensive. However, vesiculation does not alter the distribution of the stain, the vesicles remain dark in contrast to the brightly fluorescing surrounding cytoplasm.

c) DiOC₆

DiOC₆ enters the onion epidermal cells but not the tomato hair cells, presumably because of the hairs' very thick cell walls. DiOC₆ binds to a nonmotile reticulum in the cortical cytoplasm of onion epidermal cells (Figs. 34 and 36). Within this reticulum there are broad flat regions which occur at some intersections of the connecting

elements of the reticulum. The fluorochrome also binds to long fine strands which lie in bundles within the streaming cytoplasm (not shown in figures here, but see Quader and Schnepf, 1986). The connecting elements in the stationary reticulum, and the fine strands in the streaming cytoplasm, are both resolved at a diameter of about 0.2 - 0.4 μm , while the vacuoles of the MVS, which are unstained, were about 1.4 μm in diameter (Fig. 33). No staining is observed with the control solution, 0.1% ethanol. When 2.5% glutaraldehyde is added to these DiOC₆-treated cells, there is no change in the distribution of the stain, or the morphology of the cortical reticulum (Fig. 36) and the fine DiOC₆-positive strands. For example, Fig. 36 shows the same cell as in Fig. 34 treated with 2.5% glutaraldehyde. Although the plane of focus is slightly out of alignment with Fig. 34, the same connecting elements and sheets can be located. In contrast, the MVS within the same cell does vesiculate during the same fixation.

Fig. 31. An onion epidermal cell stained with neutral red and viewed with phase-contrast. The central vacuole has accumulated neutral red (NR), but no dye is found in MVS-derived vesicles (v) (600X).

Fig. 32. A tomato petiolar hair cell stained with uranin. The vacuoles of the MVS are observed as fine dark lines outlined by the intervening fluorescent cytoplasm (vacuole is bracketed by arrows) (560X).

Figs. 33-36. An onion epidermal cell first stained with DiOC₆ and then fixed with 2.5% glutaraldehyde. The cell is viewed at one plane of focus with phase contrast in Figs. 33 and 35, and at a second plane of focus with epifluorescence in Figs. 34 and 36. The plane of focus in Figs. 33 and 35 is more towards the cell interior than the plane of focus in Figs. 34 and 36. The MVS in the unfixed DiOC₆-stained cell consists of long phase-bright vacuoles (arrows in Fig. 33), quite different from the DiOC₆-positive cortical reticulum which appears as bright lines on a dark background (Fig. 34). The cortical reticulum is made up of thin connecting elements (arrows) joining broad flat DiOC₆-positive sheets (s). After the same cell has been fixed, the vacuoles of the MVS break up into spherical vesicles of various radii (arrow in Fig. 35). The ER is unaffected by fixation (Fig. 36) and, although the plane of focus is slightly out of alignment with Fig. 34, the same connecting elements (arrows) and sheets (s) can be located (800X Figs. 33 and 35, and 1600X for Figs. 34 and 36).

2) MVS-cytoplasmic associations

a) vacuolar motility

In both the hair cells and the onion epidermal cells, elements of the MVS most often move along the longitudinal axis of the cell in the peripheral cytoplasm (e.g. Figs. 37-39), or in the strands crossing the central vacuole. The individual MVS vacuoles are often surrounded by phase-dark mitochondria-rich cytoplasm which moves either with or independently of the MVS. The vacuoles themselves may sometimes be observed moving along phase-dark filaments. Vacuoles within one group can advance at speeds ranging from 1 to 7 $\mu\text{m/s}$, as calculated from several time-lapse photomicrographic time series. Mitochondria often move along the periphery of a vacuole at higher speed (in the range of 12 $\mu\text{m/s}$), occasionally shifting laterally to associate with an adjacent vacuole. These mitochondria frequently pass beyond a group of vacuoles and continue along the phase-dark filament. A concentration of the MVS is always associated with the nucleus, moving around it (Figs. 38-40) along indentations in the nuclear surface. In the hair cell, the nucleus occupies a stationary position at about the midpoint of the hair cell's length, and the MVS tends to pile up on the side of the nucleus which meets an oncoming stream of cytoplasm.

Cells viewed immediately after preparation are more likely to have vigorous streaming and an active MVS. Some cells can be left floating in water for up to 2h and still appear healthy, but the condition of most cells deteriorates with time; cytoplasmic streaming slows and, particularly in the hair cells, the peripheral streams become skewed away from the cell's longitudinal axis. After streaming stops, the MVS gradually vesiculates. Cells damaged mechanically, for example by excessive coverslip pressure, immediately show vesiculation of the MVS system.

Figs. 37-39. Phase contrast photomicrographs recorded at 15 s intervals following the movement of cytoplasmic vacuoles in a tomato hair cell. A large group of vacuoles (arrow) have moved past a stationary chloroplast (C) (1540X).

Figs. 40-42. A series of phase contrast photomicrographs recorded at 10 s intervals showing motile vacuoles moving past the nucleus in an onion epidermal cell. In Fig. 40 a loop-shaped vacuole of the MVS (arrow) is shown on the surface of the nucleus (N). By Fig. 41 the loop-shaped vacuole has moved towards the right side of the field, and the loop has extended. In Fig. 42 the loop is slightly out of the plane of focus, but has moved to the right edge of the nucleus (1300X).

b) centrifugation

Hair cell cytoplasm is stratified by centrifugation (Fig. 43) such that chloroplasts, nucleus, MVS, and the central vacuole are arranged from most to least dense, respectively. The MVS is not vesiculated by centrifugation, but the vacuoles are closely appressed to each other, so much so that vacuoles may be seen in optical cross-section as small circular profiles (Fig. 43). The central vacuole continues to occupy most of the cellular volume. Not all of the cell contents are stratified. A cortical layer of non-streaming cytoplasm retains its position, bounded on its inner surface by the tonoplast of the central vacuole, and on its outer surface by the plasmalemma. Rapidly streaming cytoplasm (including mitochondria) which usually occupies space between the cortical cytoplasmic layer and the tonoplast appears to be mostly displaced by the centrifugation treatment. However, when cells are viewed immediately after centrifugation, some mitochondria can be observed moving very slowly along fine phase-dark filaments in the stationary cortical cytoplasm (Fig. 44). During recovery, vacuoles can also be observed moving along these filaments. Some chloroplasts also remain attached to the cortical cytoplasm during centrifugation (Fig. 44).

Within 1-2 h the centrifuged cells recover pre-centrifugation organization (Figs. 45-49). This recovery process is initially recognized by vigorous streaming in the displaced cytoplasm and the movement of cytoplasm and MVS towards the centripetal pole of the cell (Figs. 45 and 46; Figs. 48 and 49). This is followed by movement of the nucleus towards the opposite (centripetal) endwall (Fig. 47). As mentioned earlier, the nucleus always occupies a position roughly midway along the cell. In the recovering cell, the nucleus moves back to this pre-centrifugation position (Fig. 47). Vacuoles, surrounded by phase-dark cytoplasm, move along fine filaments which also

support moving mitochondria. Individual vacuoles often extend beyond a group of vacuoles and become visibly longer and thinner (Fig. 49). I could find no differences in the overall recovery pattern in cells with different centrifugation treatments. However, I did observe changes in nuclear movement when centrifugation forces were varied.

Figs. 43-49. Hair cells centrifuged, mounted in distilled water and viewed with phase contrast optics. In all of these figures, the centrifugal end of the cell is oriented towards the bottom of the page. Therefore, the centripetal movement of the organelles and cellular components is always represented as upwards.

Fig. 43. Tomato hair cell centrifuged at 8400 g for 10 min, mounted in distilled water and viewed with phase contrast. Chloroplasts (C) occupy the most centrifugal position followed by the nucleus (N), the MVS, which appear in optical section as small circles (bracketed by arrows), and the central vacuole (V). Mitochondria cannot be reliably identified in this image (1460X).

Fig. 44. A nonmotile cortical cytoplasmic layer is not shifted by centrifugation. Some chloroplasts (C) remain attached to this layer, and some mitochondria (M) can be observed moving along fine dark filaments (bracketed by arrows). Some out of focus wax plates (w) on the cell wall are also visible (1900X).

Figs. 45-49. Within 15 min the initiation of cellular recovery is recognized by the centripetal movement of cytoplasm (Figs. 45 and 46), MVS vacuoles (Figs. 48 and 49), and the nucleus (Fig. 47). In Figs. 45 and 46 the advancing front of cytoplasm (large arrow) can be compared to the position of stationary, slightly out of focus chloroplasts (small arrows) (840X). Figs. 48 and 49 show the movement of vacuoles (arrows) (time interval of 10 s) relative to a stationary chloroplast (C) (1740X). Nuclear movement is slower; Fig. 47 shows the position of a nucleus about 30 min after this cell was centrifuged. Note cytoplasmic streams (S) which radiate away from nucleus (410X).

Nuclear movement is a clear and easily measured feature of cellular recovery, and was used as an indicator of relative recovery rates. By following nuclear movement during cellular recovery, and plotting nuclear velocity against time, I could easily compare patterns of nuclear movement from one treatment to the next (samples of such plots are given in Figs. 50 and 51). The plots show that at some point in the recovery process, the nucleus reaches a maximum velocity ($v_{\max}$). I calculated the average $v_{\max}$ for nuclear movement in seven cells recovering from either the weak or strong centrifugation treatment. I was also interested in the time it took the nuclei to reach their maximum velocity, so I also determined the mean time to reach $v_{\max}$ (t_v) following each centrifugation treatment. There does not seem to be a significant difference in the time at which this maximum nuclear velocity is reached (indeed, the t_v value has a wide range after both the weak and the strong treatments), but the value of $v_{\max}$ is significantly affected by changing the centrifugal force used to displace the cytoplasm. The average nuclear $v_{\max}$ for cells recovering from the strong treatment is about half that for cells following the weak treatment (Table 5).

Table 5. A comparison of the average time to reach maximum nuclear velocity (t_v), and average maximum nuclear velocity ($v_{\max}$) following centrifugation treatments A and B

treatment	t_v (min)	$v_{\max}$ ($\mu\text{m min}^{-1}$)
weak	57.2 ± 32.4	$3.37 \pm 1.63^*$
strong	45.6 ± 26.7	$1.85 \pm 0.77^*$

*significantly different ($p < 0.05$), $n = 7$ for each treatment (ANOVA).

Figs. 50 and 51. Sample plots showing the variation of nuclear velocity with time in hair cells recovering from the weak centrifugation treatment (Fig. 50, 4100g for 7 min) and the strong treatment (Fig. 51, 8400g for 14 min).

50

51

b) cytoskeletal associations:

Both colchicine and nocodazole have no effect on streaming or cellular morphology. In contrast, cytochalasin D inhibits streaming within a few minutes, although the time required for inhibition depends upon concentration. For example, 20 μM cytochalasin D will arrest streaming within 3 min, but requires up to 15 min at 2 μM . Cytochalasin D not only inhibits cytoplasmic streaming, it also changes cellular organization. A phase-dark component of the cytoplasm, rich in mitochondria, peels away from the MVS vacuoles to form aggregates (Fig. 52) leaving a few isolated mitochondria fastened to the vacuoles. This process is separate from the osmotic effect of the DMSO, which causes the vacuoles to thin and fold (Fig. 15). Cytoplasm within these aggregates continues to show disorganized streaming. After the separation of this cytoplasmic component, the vacuolar boundaries often appear more clearly defined and in higher contrast than in untreated cells (Fig. 53). However, the vacuoles are not vesiculated by treatment with cytochalasin D (Fig. 53). The separation of the aggregates of cytoplasm from the vacuoles is irreversible even when the drug is washed out and cells left for 1h or more. Cytochalasin D also completely inhibits cellular recovery after centrifugation, whether it is applied before or after centrifugation. Post-centrifugation recovery is unaffected by the two microtubule-disrupting drugs. Additionally, application of cytochalasin D before centrifugation does not change the pattern of stratification (Fig. 54).

Staining with rhodamine-phalloidin reveals that hair cell actin is arranged as filaments of various diameters (0.4 to 4 μm) oriented parallel to the cell's longitudinal axis (Fig. 55). Many filaments converge on the nucleus, which appears covered with actin. When cells are pre-treated with cytochalasin D, no actin-staining is observed. During centrifugation, nuclear-associated actin is displaced with the nucleus, and

reorganizes with cellular recovery (Figs. 56 and 57). Since it is necessary to kill the cell to observe the actin, it is impossible to determine if the pattern of actin organization after recovery is the same as pre-centrifugation patterns.

Figs. 52 and 53. Tomato hair cell treated with 5 μ M cytochalasin D and viewed with phase contrast optics. A component of the cytoplasm peels away from the vacuoles and collects into phase-dark aggregates (A). The boundaries between vacuoles often become more clearly defined (see arrow in Fig. 53) (Fig. 52, 1300X; Fig. 53, 1400X).

Fig. 54. This hair cell (viewed with phase contrast optics) was treated with cytochalasin D before centrifugation, but still has a pattern of stratification similar to controls. The figure shows the relative placement of the central vacuole (V), the MVS (again seen as small circles in optical section, arrows), and some of the chloroplasts (C). The nucleus and most of the chloroplasts are not in this plane of focus, which was chosen to illustrate the circular profile of the MVS vacuoles when viewed in optical section. The cytochalasin D treatment completely inhibits post-centrifugation recovery (910X).

Figs. 55 - 57. Hair cells stained for actin with rhodamine-conjugated phalloidin and viewed with epifluorescence. Fig. 55 shows the nucleus (N) and longitudinally oriented actin filaments (arrows) in an uncentrifuged cell. Some of the actin filaments are deposited with the nucleus during centrifugation (small arrow), while others (large arrow) remain anchored in the undisplaced cortical cytoplasm (Fig. 56). As the cell recovers after centrifugation, the nucleus becomes central in a reorganizing actin framework (Fig. 57, cell stained at 30 min after centrifugation) (Figs. 55-57, 260X).

Discussion

1) Vital dyes

a) Neutral Red

The staining reaction in the central vacuole of *Allium* (a dull brick-red with no dye aggregates) indicates that an ion-trap mechanism is at work, which concurs with results from Stadelmann and Kinzel (1972). The MVS of the onion epidermal cells may have a more alkaline pH than the central vacuole, does not contain the necessary phenolic compounds to chemically bind the vital dye, or has a membrane which is impermeable to the dye. The critical point is that the two vacuolar spaces are evidently separate, and perhaps functionally distinct. Palevitz *et al.* (1981) described a vacuolar system in *Allium* guard cells which in some respects resembles the MVS shown here, in that it was composed of long, motile, pleomorphic vacuoles. The vacuolar system observed by Palevitz *et al.* accumulated neutral red but also contained compounds which autofluoresced when excited with blue light. These autofluorescent compounds could have reacted with the neutral red, trapping it within the vacuolar space. *Bryopsis* also accumulates neutral red within a secondary vacuolar system very similar to the MVS (Menzel and Schliwa 1986), but it is impossible to tell which of the above mechanisms (ionic or chemical) are responsible. Contents of vacuoles vary from cell type to cell type, adapting to necessary functions, and the lack of neutral red accumulation in the MVS studied here does not make the system any less vacuolar in nature. The tomato petiolar hair cell does not accumulate neutral red in any cellular compartment but the hair cells exclude other reagents as well (e.g. DiOC₆) so neutral red is not peculiar in this regard. Wenzel and McCully (1991) had similar problems when attempting to stain corn root cortical and epidermal cells. Beyond a few

centimetres from the root tip, these cells did not accumulate neutral red, even though streaming was evident. The inability of the neutral red to enter the hair cells makes it an inappropriate dye for distinguishing between MVS and the central vacuole in these cells. However, two observations reported in Mersey (1977) suggest that the MVS and the central vacuole of the hair cell have different contents. 1) Crystals form in the central vacuole of an unhealthy hair cell but not in the vacuoles of the MVS. The crystals, obvious optically, are also apparent in electron micrographs as a precipitate present only in the large central vacuole. 2) When hair cells are fixed in solutions of high calcium concentration (e.g. 0.1 M CaCl_2) and prepared for electron microscopy, the MVS-derived vesicles contain a fibrillar precipitate not present in the central vacuole. Both of these observations indicate that the two vacuolar spaces respond differently to the same environment. It is difficult to carry these observations beyond this initial distinction, but a preliminary stance that the MVS and the central vacuole in the hair cells constitute separate vacuolar spaces seems justified.

b) Uranin

Staining with uranin emphasised the vacuolar nature of the MVS by contrasting it against the cytoplasmic space. This makes uranin more useful than neutral red in determining the nature of membrane forms observed in the hair cells with DIC, since the stain is able to enter the cell. Since staining with uranin results in a dark space between fine fluorescent lines (lines which appear as filaments in DIC), then the filaments cannot represent ER-tubules. The whole field would stain uranin-positive if the spaces between the filaments was cytoplasmic. Uranin did not enter either the central vacuole or the MVS indicating that the dye was either excluded from these spaces, or that they did not contain substances which react with uranin (e.g. protein). Craig (1986), reasoning from observations of cytoplasmic vesicles in electron

micrographs, postulated that in cells of pea cotyledons there may be a vacuolar system similar to the pleomorphic canalicular system (i.e. MVS) "involved in reserve protein accumulation and concentration" prior to storage in the central vacuole. I have no evidence of proteins in the MVS of hair or onion epidermal cells, but vacuoles have been adapted to a variety of uses in a range of cell types (e.g. salt accumulation, protein storage, etc.; Robinson, 1985) and it is conceivable that a secondary vacuolar system could serve a number of functions.

c) DiOC₆

The cortical reticulum stained with DiOC₆ in the onion epidermal cells was similar in appearance, placement and dimensions to the reticulum observed by Quader and Schnepf (1986) in the same cell type, and by Terasaki *et al.* (1984) in animal tissue culture cells (see their Figs. 1 and 3A). The reticulum also resembled that revealed by chlortetracycline (Figs. 24-26) indicating the role of the ER in calcium compartmentalization. The individual tubules within the reticular array and in the tubular bundles have dimensions similar to those recorded by Quader and Schnepf (1986). Their onion epidermal cells, like the ones shown here, had DiOC₆-stained tubules which appear as 0.4 or 0.5 μm in diameter in fluorescent micrographs, but were measured as 0.1 μm in electron micrographs (Quader *et al.*, 1987). The vacuoles of the MVS, however, are of a much greater diameter (1.4 μm or larger in Fig. 33) and may bend and realign, or gather into globular masses. Most importantly I found that the components of the reticulum, and the fine strands within the motile cytoplasmic phase, survive both long-term exposure to blue excitation illumination (the MVS vesiculates quickly under this treatment), and chemical fixation. Similarly Terasaki *et al.* (1984) found the DiOC₆-stained reticulum to be stable with glutaraldehyde or potassium permanganate fixation. A very important point to re-emphasize here is that

Quader and Schnepf (1986) could locate the preserved 0.1 μm diameter tubules of the ER in electron micrographs but the MVS can be found only as 1-6 μm diameter spherical cytoplasmic vacuoles.

The bounding membrane of the MVS did not appear to stain with DiOC₆, but I propose that this may be an observational artefact, since DiOC₆ is expected to partition into all of the cellular membranes (Terasaki, 1989). I believe the greater intensity of the ER-staining might be due to the volume of the membrane within a given optical sample. When the cell is viewed through the microscope, you are sampling a specific volume, determined by the depth of field of the objective. A 100X oil immersion objective has a depth of field on the order of 0.2 μm (Slayter, 1970), roughly equivalent to the diameter of the ER tubules (Fig. 58). I would expect more ER membrane than MVS membrane in a 0.2 μm thick sample volume, giving the ER tubule an apparently greater fluorescence intensity. Although the MVS membrane may not be DiOC₆-positive, this dye's apparent specificity for ER in plant cells may be artefactual.

Fig. 58 A comparison of the membrane volume sampled when viewing part of a MVS-vacuole (a) and an ER tubule (b) with a 100X objective having a depth of field of approximately 0.2 μm .

The pattern of staining observed with DiOC₆ is very different from that reported for Auramine O, a proposed fluorescent probe for endomembranes (Harris and Gates, 1984). The distinct reticulum visible with DiOC₆ is not apparent in the figures

provided by Harris and Gates. Harris and Gates misinterpreted their fluorescence microscopy, and incorrectly compared tissue in fluorescence micrographs with tissue post-fixed with a zinc iodide-osmium tetroxide (ZIO) complex for transmission electron microscopy. Although the ZIO post-fixation was intended to reveal ER, other work has shown that this technique is non-specific and fails to stain cells in the same tissue uniformly (Hepler, 1981). The structure revealed by Auramine O (which they interpret as a reticulum) is quite different from the structure revealed by their ZIO post-fixation procedure. The open spaces in the fluorescing reticulum are about an order of magnitude larger than the open spaces revealed in electron micrographs (see their Figs. 7 and 8). The fluorescing reticulum is also qualitatively different from the ER shown in the electron micrographs. The Auramine O-positive structure was coarse and granular, quite unlike the smooth lace-like network in the electron micrographs. It is interesting that the one electron micrograph that does illustrate a structure similar to that in the fluorescence micrographs (their Fig. 4) is of a cell quite obviously destroyed by poor fixation. The cytoplasm is coarse and granular and the large perinuclear space is indicative of coagulant fixation (O'Brien and McCully, 1981). This preparation would surely make most organelles and membrane systems unrecognizable. The structure revealed by the Aurimine O is typically observed in dead, severely damaged cells (I have observed similar patterns with uranin in dead cells). When I attempted to repeat Harris and Gates' procedure, I found that I could only get Aurimine O into dead cells, and that the dye would not enter living cells. I do not understand their comments about staining being more intense in more metabolically active cells. I believe that Aurimine O does not represent a useful stain for plant endomembrane systems because of its apparent unreliability as a fluorescent probe for structure in living cells.

2) MVS-cytoplasmic associations

Actin in the tomato petiolar hair surrounds the nucleus and is longitudinally oriented (Fig. 55, see also Parthasarathy *et al.*, 1985), roughly parallel with the MVS and cytoplasmic streaming. The actin around the nucleus may support the movement of vacuoles and mitochondria along the nuclear surface. One would predict that actin is also closely associated with the MVS vacuoles, since mitochondria are observed travelling along the vacuoles. Actin has been found to be closely associated with small, cylindrical vacuoles in *Drosera* tentacle cells (Lichtscheidle *et al.* 1990) and pollen tubes (Tiwari and Polito, 1988). The phase-dark filaments which direct the movements of organelles (Fig. 44) are probably actin filaments, and have been observed in other cells (O'Brien and McCully, 1970; Parthasarathy and Mühlethaler, 1972). Mersey (1977) has already shown that disruption of actin with cytochalasin B inhibits cytoplasmic streaming and MVS movement in hair cells. Therefore, it was not surprising that treatment with cytochalasin D produced similar effects. More interesting than the inhibition of vacuolar movement and cyclosis is that the cytochalasin treatments drastically alter MVS-cytoplasmic associations, indicating the role of actin in the organization of the MVS.

Palevitz *et al.* (1981) found that the MVS-like vacuoles in developing onion guard cells were also drastically affected by cytochalasin B, which broke down the fine tubular network into several globular cisternae. However, Palevitz *et al.* viewed the effects of the cytoskeletal drug by fluorescence microscopy, a technique known to vesiculate cylindrical vacuoles. Therefore, the globular cisternae observed by the authors may have been vesicles gathered into tight groups. In the absence of fixation, mechanical damage or fluorescence microscopy, the cytochalasin-induced

rearrangement of the MVS in hair cells does not involve vesiculation. This is particularly interesting in view of the finding that cytochalasin D does not alter intracellular calcium levels (Keifer *et al.*, 1992) (however, in human leukocytes cytochalasin D has been found to increase calcium concentrations by about 0.1 μM (an increase which is not pathological). Although cytoplasmic streaming is a calcium-dependent process, the inhibition of streaming by cytochalasin D is thought to be actin-specific (Lloyd, 1988). According to the proposal put forward in Chapter 2 about the dependence of vesiculation on the cellular ionic environment, the finding that vacuoles are not vesiculated during treatment with cytochalasin D indicates that the drug does not drastically alter calcium levels (at least not to pathological levels).

The phase-dark aggregates which peel away from vacuoles after cytochalasin treatment (Fig. 52) likely contain segments of actin filaments, and associated organelles (e.g. mitochondria) (Schliwa 1982). Lancelle and Hepler (1988) used rapid freeze-fixation and freeze-substitution to observe the effects of cytochalasin B and D on the ultrastructural organization of *Nicotiana* pollen tubes. They found that the actin in treated pollen tubes gathers into massive bundles with no consistent orientation to the pollen tube axis. The aggregates observed here may contain such large actin bundles even though they do not stain with rhodamine-phalloidin. Lancelle and Hepler point out that cytochalasin-bound actin does not bind phalloidin as well as untreated actin. A major difference between the pollen tubes observed by Lancelle and Hepler and the hair cells observed here was that the pollen tubes recovered their original organization and activity after drug removal (within ten minutes), but the hair cells did not (even after an hour of rinsing). The hair cells were not more severely treated than the pollen tubes, and their inability to recover after drug removal is not explained by the larger size of the hair cells (about two orders of magnitude larger in radius than the pollen tubes), since

larger cells have been observed to recover from cytochalasin treatments (e.g. *Acetabularia* cells 2-3 mm in length, Koop and Kiermayer, 1980). The irreversibility of the cytochalasin effects in the hair cells may be due to their thick cell walls inhibiting proper removal of the drug.

In contrast to the importance of actin in MVS orientation and morphology, microtubules seem not to be associated with the vacuoles. Palevitz *et al.* (1981) also found that colchicine did not have any immediate effects on the vacuolar system in *Allium* guard cells; changes could only really be observed after 2-4 h and some treatments were observed for up to 24 h. The longer treatments were found to decrease the reticular nature of their vacuolar system. Salitz and Schmitz (1989) demonstrated that nocodazole and oryzalin (another microtubule disrupting drug) inhibited cytoplasmic streaming only in treatments lasting over 3 h. I did not allow treatments to continue longer than 2 h, considering that any effects produced past this time were difficult to discriminate from general cellular fatigue. Since the microtubule-disrupting drugs required such long times to have any effect on MVS movement and organization, it seems that the vacuoles of the MVS are not closely associated with microtubules, unlike ER which has been shown to be interdependent with both actin and microtubules (Terasaki *et al.* 1986; Kachar and Reese, 1988). Parallel observations with microtubule labels (using immunofluorescence) are required to determine the pattern of microtubule organization relative to the MVS. However, these results indicate that actin, not microtubules, are important in the organization of the MVS. Results from centrifugation treatments show that actin is also important in the organization of the entire cell.

Centrifugation of the hair cells stratifies cytoplasmic components according to their relative densities. The chloroplasts, occupying the most centrifugal position, are

densest, followed closely by the nucleus, which was often embedded within the group of chloroplasts. Centrifugation of different cell types has shown that the chloroplasts and nucleus are often found in the denser cell fraction (Moroz, 1984; Beams, 1949). Other work has also shown that the nucleus may become distended by centrifugal forces, forcing the nucleolus to the centrifugal pole of the nucleus (Bouck, 1963). I did not observe this effect, but the centrifugal forces used in my treatments were not as great as those used by Bouck (1963) and were insufficient to produce stratification within the nucleus. Vacuoles are typically the least dense organelles (Bouck, 1963), but earlier literature seldom distinguishes between different types of vacuolar spaces. It is interesting that the MVS is more dense than the central vacuole, and this result suggests the MVS contents may be less dilute or have a greater inherent specific gravity. The greater density of the MVS supports the earlier contention that the small cytoplasmic vacuoles are distinct in their nature from the central vacuole in the tomato hair.

The cortical cytoplasm and actin filaments remaining *in situ* after centrifugation seem to be important in initiating and organizing cellular recovery. The first cytoplasmic streams which form after centrifugation, and are important in the centripetal transport of organelles and bulk cytoplasm, are probably laid out along these actin filaments. It is along these streams that the MVS moves out with the bulk cytoplasm and quickly becomes redistributed throughout the cell. Bouck (1963) could not directly observe the recovery of his pea root meristematic cells (he used only electron microscopy), but he did note that small cytoplasmic vacuoles were redistributed within 2h after centrifugation. It is not surprising that the MVS is quickly reorganized, since the small vacuoles are less dense than the other organelles (except for the central vacuole). Denser organelles (e.g. the nucleus and chloroplasts) require more time to

move into pre-centrifugation positions, not only because they have further to go, but also because more work is required to move them.

Cellular recovery following centrifugation was found to be actin-dependent, since treatment with cytochalasin D (both before and after centrifugation) completely inhibited the recovery process. Therefore, the residual actin observed in the cortical cytoplasm of centrifuged cells is probably important in the organization of new cytoplasmic streams and subsequent cellular recovery. Quader *et al.* (1987) did not observe residual actin in their onion epidermal cells following centrifugation, although they did find that reorganization of the actin framework preceded cellular recovery. However, their centrifugal accelerations were considerably greater than my treatments (30,000 g compared with 4,000 - 8,000 g), and their cells took longer to recover. The milder nature of my treatments, while stratifying the cells, still permitted some of the actin framework closely associated with the cortical cytoplasm to remain intact, giving the hair cell something of a headstart in its recovery.

During cellular recovery, vacuoles of the MVS begin to move with those first streams which carry cytoplasm and mitochondria towards the centripetal pole, presumably along the actin filaments remaining after centrifugation. Chloroplasts are also transported along these pathways. Although chloroplast movement has been found, in some systems, to be both microtubule and actin-dependent (Menzel and Schliwa, 1986), chloroplast movement in the hair cells seems strictly actin-dependent. While chloroplast movement might be modelled simply as a myosin coated bead moving along an actin cable (Sheetz and Spudich, 1983), nuclear movement seems more complicated. Instead, any nuclear translocation requires major reorganization of the actin framework (see Figs. 56 and 57). The nucleus does not simply ride upon an actin track, but is instead encased in an actin basket, and this basket is somehow

actively positioned relative to the entire actin framework. This model is consistent with observations from other systems (e.g. suspension culture cells of carrot, Traas *et al.*, 1987; pollen tubes, Heslop-Harrison and Heslop-Harrison, 1989; *Allium* epidermal cells, Mineyuki and Palevitz, 1990). For example, in *Narcissus pseudonarcissus* and *Endymion nonscriptus* the vegetative nucleus is very elongated (about 50 μm in length), but contracts considerably (to about one half its length) after the pollen tube is treated with cytochalasin D (Heslop-Harrison and Heslop-Harrison, 1991). Heslop-Harrison and Heslop-Harrison propose that the reduction in nuclear length is due to a release of tension when surrounding actin is removed. When the pollen tube is left to recover from the cytochalasin treatment, the nucleus re-elongates and moves into a newly grown secondary pollen tube. In control pollen tubes, the vegetative nucleus is always about 140 μm away from the growing tip. In pollen tubes which have recovered from the cytochalasin treatment, the distance of the vegetative nucleus from the new growing tip is not significantly different from the controls. This indicates that the vegetative nucleus, like the nucleus in the hair cells, is kept within a stable position relative to a dynamic framework. This ability of the cell to include the nucleus within the general organization of the actin framework may explain how the hair cell nucleus was repositioned after centrifugation. As the actin framework reorganized, the nucleus was simply carried along within its actin basket. A similar model has been suggested for the movement of chloroplasts in cells of the algae *Caulerpa* (Menzel and Elsner-Menzel). Mineyuki and Palevitz (1990) suggest that the actin sheath that is commonly observed surrounding nuclei may be important in directing migrating nuclei to specific sites in dividing plant cells. The large scale reorganization of actin and surrounding cytoplasm which is responsible for nuclear movement and positioning may also explain the differences in maximum nuclear velocities following the two different centrifugation treatments. One would expect that the two types of centrifugation treatment (weak and

strong) would have different qualitative and quantitative effects on actin organization. It is difficult to surmise the causes behind the observed differences in nuclear velocity, but the explanation will need to include both a mechanical appreciation of the relative masses of displaced cytoplasm as well as an assessment of different actin organizations. One begins to appreciate that organelles are not placed independently to each other, but instead are positioned according to patterns established at higher levels of organization. It is interesting and useful that the movement and distribution of the MVS reflects to a large extent the underlying pattern of the actin framework which directs the organization of the vacuolar system and the cell.

General Discussion

At the beginning of this work, I identified a problem concerning the identification and acceptance of an extensive motile membrane-bounded system in plant cells. Having established a working name, the motile vacuolar system (MVS), based upon accepted definitions and dimensions of vacuoles, I set out the goals for this thesis. The first was to discover how the identity of the MVS could generate such confusion and contention. I discovered that the primary source of confusion could be traced back to a series of publications in the 1960's where different authors interpreted and identified the same system in widely different ways. Their inconsistent and vague identifications arose chiefly through inaccurate interpretation of their photomicrographs, and improper use of background literature. Inaccurate interpretation of photomicrographs (very similar to those shown in Figs. 6 and 7) was usually caused by a difficulty in assessing the system's three-dimensional structure. In addition to a literature littered with conflicting interpretations, is the problem that the MVS does not survive chemical fixation. During fixation, the vacuoles of the MVS break up into vesicles. This phenomenon has been recognized and studied (Mersey and McCully, 1978), but not quantitatively studied with a view to preparing a predictive model of the process. This constituted a second goal.

A mathematical analysis of vesiculation has revealed important physical properties of the MVS and membrane cylinders in general. Specifically, the mathematical study has provided a measurement for the hydraulic conductivity of the MVS membrane. Having determined this value it was possible to predict vesiculation patterns given the osmotic conditions during fixation. More importantly, the appreciation of the relationship between relative volume changes and surface area to volume ratios provided the necessary theoretical tools to re-interpret data from other

work. Indeed, the general application of these concepts has led me to formulate an alternative model for flow through sieve tubes, work not included in this document. Similarly, the experiments with myelin forms and chlortetracycline-staining of onion epidermal cells suggests that the physical cause behind the vesiculation process is one shared by fluid cylinders of all types, whether they be strings of taffy, or water on a spider's web. The mathematical model is predictive only if one interprets ambiguous structures in photomicrographs correctly. Judging the system's three-dimensional shape is actually helped by the process of vesiculation. The breakdown of the vacuoles makes clear their shape and size, and disallows alternative interpretations of the system's structure (e.g. vacuolar boundaries interpreted as filaments or ER tubules). Distinguishing the MVS from other membrane-bounded systems, while examining the associations between the MVS and the surrounding cytoplasm represented the third goal of this study.

The MVS is a vacuolar space separate from the large central vacuole in both onion bulb-scale epidermal cells and tomato petiolar hair cells. In the onion cells, the central vacuole takes up neutral red while the MVS does not. Centrifugation of the hair cells has shown that the MVS is more dense than the central vacuole in these cells, indicating that their contents are distinct from each other. Uranin clearly identifies the system as vacuolar, because the vacuoles appear as dark shapes against a fluorescing, uranin-positive cytoplasmic background. Although the MVS is disrupted by chemical fixation, the cortical ER in onion epidermal cells is not (as evidenced by the appearance of DiOC₆-stained cells after fixation). This is an interesting difference between the two membrane systems, and it is proposed that the relative stability of the MVS and ER during fixation may be due to their possessing contents of different viscosities (this goes back to the general properties of fluid cylinders discussed above). The motile

vacuoles are closely associated with actin, which directs the global distribution of the MVS, and also seems to be an important intermediate in the association between the vacuoles and surrounding fluid cytoplasm.

While the modelling work has shown that the vacuoles respond to changes in cellular water potential, thus indicating a possible role in cellular water homeostasis, more work is required which would experimentally examine the function of the MVS in cellular water relations. This might be done by tracking changes in MVS dimensions (specifically vacuolar radius), while varying the water potential of the cell's environment. At the same time the water potential of the central vacuole could be monitored and the relative response of the larger vacuole and the MVS compared. This would be accomplished more easily in very large cells, like those found in *Bryopsis*, where a pressure probe could be used to directly measure the water potential of the central vacuole. It would be interesting to determine how rapidly the MVS would respond to the cell's environment (as judged by changes in vacuolar surface area to volume ratios) compared with the central vacuole. Alternatively, other possible functions of the system could be explored. This might best be done with motile vacuolar systems in other cell types where the elongated vacuoles have been shown to have identifiable contents. It has not been possible to histochemically reveal the contents of the MVS in the hair cells or onion epidermal cells (although contents have been observed in the MVS in hair cells where calcium was included in the fixative solution). However, this is not true for other cell types. For example, the MVS in *Bryopsis* accumulates neutral red (Menzel and Schliwa, 1986), and long cylindrical vacuoles in *Saprolegnia* hyphae have electron-opaque contents (Heath *et al.*, 1985). An investigation of these contents and their distribution through the cell by the vacuoles may reveal that an MVS, while being a common structural component of plant cells, is

adapted to a variety of functions. Such functions for systems of elongated motile vacuoles will probably be discovered as work with cryopreservation continues to reveal these systems intact in a variety of cell types.

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Appendix 1: Computer program for simulating vesiculation and sample output.

Computer program used to simulate vesiculation.

```

10,000  PROGRAM THE SIMULATED TUBULIFEROUS VESICULATION
20,000  DIMENSION TUBE(100),TIME(100),VOLUME(100),AREA(100),PERCENT(100)
30,000  TIME=0
40,000  DO 100 I=1,100
50,000    TUBE(I)=0
60,000    VOLUME(I)=0
70,000    AREA(I)=0
80,000    PERCENT(I)=0
90,000  100 CONTINUE
100,000  PRINT*,'TUBULIFEROUS VESICULATION'
110,000  PRINT*,'NUMBER OF TUBES: 100'
120,000  PRINT*,'NUMBER OF TUBES: 100'
130,000  PRINT*,'NUMBER OF TUBES: 100'
140,000  PRINT*,'NUMBER OF TUBES: 100'
150,000  PRINT*,'NUMBER OF TUBES: 100'
160,000  PRINT*,'NUMBER OF TUBES: 100'
170,000  PRINT*,'NUMBER OF TUBES: 100'
180,000  PRINT*,'NUMBER OF TUBES: 100'
190,000  PRINT*,'NUMBER OF TUBES: 100'
200,000  PRINT*,'NUMBER OF TUBES: 100'
210,000  PRINT*,'NUMBER OF TUBES: 100'
220,000  PRINT*,'NUMBER OF TUBES: 100'
230,000  PRINT*,'NUMBER OF TUBES: 100'
240,000  PRINT*,'NUMBER OF TUBES: 100'
250,000  PRINT*,'NUMBER OF TUBES: 100'
260,000  PRINT*,'NUMBER OF TUBES: 100'
270,000  PRINT*,'NUMBER OF TUBES: 100'
280,000  PRINT*,'NUMBER OF TUBES: 100'
290,000  PRINT*,'NUMBER OF TUBES: 100'
300,000  PRINT*,'NUMBER OF TUBES: 100'
310,000  PRINT*,'NUMBER OF TUBES: 100'
320,000  PRINT*,'NUMBER OF TUBES: 100'
330,000  PRINT*,'NUMBER OF TUBES: 100'
340,000  PRINT*,'NUMBER OF TUBES: 100'
350,000  PRINT*,'NUMBER OF TUBES: 100'
360,000  PRINT*,'NUMBER OF TUBES: 100'
370,000  PRINT*,'NUMBER OF TUBES: 100'
380,000  PRINT*,'NUMBER OF TUBES: 100'
390,000  PRINT*,'NUMBER OF TUBES: 100'
400,000  PRINT*,'NUMBER OF TUBES: 100'
410,000  PRINT*,'NUMBER OF TUBES: 100'
420,000  PRINT*,'NUMBER OF TUBES: 100'
430,000  PRINT*,'NUMBER OF TUBES: 100'
440,000  PRINT*,'NUMBER OF TUBES: 100'
450,000  PRINT*,'NUMBER OF TUBES: 100'
460,000  PRINT*,'NUMBER OF TUBES: 100'
470,000  PRINT*,'NUMBER OF TUBES: 100'
480,000  PRINT*,'NUMBER OF TUBES: 100'
490,000  PRINT*,'NUMBER OF TUBES: 100'
500,000  PRINT*,'NUMBER OF TUBES: 100'
510,000  PRINT*,'NUMBER OF TUBES: 100'
520,000  PRINT*,'NUMBER OF TUBES: 100'
530,000  PRINT*,'NUMBER OF TUBES: 100'
540,000  PRINT*,'NUMBER OF TUBES: 100'
550,000  PRINT*,'NUMBER OF TUBES: 100'
560,000  PRINT*,'NUMBER OF TUBES: 100'
570,000  PRINT*,'NUMBER OF TUBES: 100'
580,000  PRINT*,'NUMBER OF TUBES: 100'
590,000  PRINT*,'NUMBER OF TUBES: 100'
600,000  PRINT*,'NUMBER OF TUBES: 100'
610,000  PRINT*,'NUMBER OF TUBES: 100'
620,000  PRINT*,'NUMBER OF TUBES: 100'
630,000  PRINT*,'NUMBER OF TUBES: 100'
640,000  PRINT*,'NUMBER OF TUBES: 100'
650,000  PRINT*,'NUMBER OF TUBES: 100'
660,000  PRINT*,'NUMBER OF TUBES: 100'
670,000  PRINT*,'NUMBER OF TUBES: 100'
680,000  PRINT*,'NUMBER OF TUBES: 100'
690,000  PRINT*,'NUMBER OF TUBES: 100'
700,000  PRINT*,'NUMBER OF TUBES: 100'
710,000  PRINT*,'NUMBER OF TUBES: 100'
720,000  PRINT*,'NUMBER OF TUBES: 100'
730,000  PRINT*,'NUMBER OF TUBES: 100'
740,000  PRINT*,'NUMBER OF TUBES: 100'
750,000  PRINT*,'NUMBER OF TUBES: 100'
760,000  PRINT*,'NUMBER OF TUBES: 100'
770,000  PRINT*,'NUMBER OF TUBES: 100'
780,000  PRINT*,'NUMBER OF TUBES: 100'
790,000  PRINT*,'NUMBER OF TUBES: 100'
800,000  PRINT*,'NUMBER OF TUBES: 100'
810,000  PRINT*,'NUMBER OF TUBES: 100'
820,000  PRINT*,'NUMBER OF TUBES: 100'
830,000  PRINT*,'NUMBER OF TUBES: 100'
840,000  PRINT*,'NUMBER OF TUBES: 100'
850,000  PRINT*,'NUMBER OF TUBES: 100'
860,000  PRINT*,'NUMBER OF TUBES: 100'
870,000  PRINT*,'NUMBER OF TUBES: 100'
880,000  PRINT*,'NUMBER OF TUBES: 100'
890,000  PRINT*,'NUMBER OF TUBES: 100'
900,000  PRINT*,'NUMBER OF TUBES: 100'
910,000  PRINT*,'NUMBER OF TUBES: 100'
920,000  PRINT*,'NUMBER OF TUBES: 100'
930,000  PRINT*,'NUMBER OF TUBES: 100'
940,000  PRINT*,'NUMBER OF TUBES: 100'
950,000  PRINT*,'NUMBER OF TUBES: 100'
960,000  PRINT*,'NUMBER OF TUBES: 100'
970,000  PRINT*,'NUMBER OF TUBES: 100'
980,000  PRINT*,'NUMBER OF TUBES: 100'
990,000  PRINT*,'NUMBER OF TUBES: 100'
100,000 PRINT*,'NUMBER OF TUBES: 100'

```


Sample output from program where $R_0 = 1.25 \mu\text{m}$, $l = 15 \mu\text{m}$, $\Psi_0 = 1 \text{ MPa}$, and $L_w = 10^{-15} \text{ m Pa}^{-1} \text{ s}^{-1}$.

```

      1.250
      15.000
1.000E+006
1.000E-009
      1.562      15.791      154.944      120.983
      1.766      15.122      120.296      76.153
      1.480      15.193      141.277      104.540
      1.388      15.367      134.014      93.007
      1.559      15.324      150.117      117.022
      1.341      15.681      132.158      98.637
      1.315      15.062      124.414      81.779
      1.471      15.348      141.873      104.363
      1.574      15.057      148.917      117.200
      1.544      15.078      145.301      111.428
y
1.014E+006 1.105E-009
A
1.010E+006 1.261E-009
Z
1.004E+006 1.228E-009
      1.126      21.892      154.944      87.267
      0.831      23.043      120.296      49.976
      0.000      0.000      0.000      0.000
      0.953      22.385      134.014      63.845
      1.124      21.258      150.117      84.356
      0.000      0.000      0.000      0.000
      0.879      22.516      124.414      54.706
      1.036      21.795      141.873      73.491
      0.000      0.000      0.000      0.000
      1.099      21.051      145.301      79.810
q
1.003E+006 1.068E-009
Z
1.004E+006 1.266E-009
      0.815      29.170      154.944      65.495
      0.000      0.000      0.000      0.000
      0.000      0.000      0.000      0.000
      0.672      21.750      134.014      45.015
      0.000      0.000      0.000      0.000
      0.000      0.000      0.000      0.000
      0.758      23.091      124.414      37.224
      0.755      29.908      141.873      53.556
      0.000      0.000      0.000      0.000
      0.818      28.287      145.301      59.394
j
1.031E+006 1.193E-009
7
1.022E+006 1.073E-009
4
1.030E+006 1.282E-009
10
1.005E+006 1.312E-009
8
1.023E+006 1.093E-009
      7      1.268      20.208      8.542      0.846      13.488      5.702
      9      1.054      13.950      4.899      0.657      8.696      3.054
      3      1.789      40.219      23.984      0.917      20.619      12.246
      30      0.559      4.356      0.855      0.452      3.343      0.656
      4      1.686      35.713      20.069      0.343      7.264      4.082
      3      1.810      11.185      24.853      0.378      9.604      5.192
      21      0.676      5.744      1.294      0.447      3.794      0.855
      199      0.239      0.715      0.057      0.104      0.311      0.025
      2      0.261      70.052      55.132      0.297      8.812      6.935
      25      0.570      4.070      0.775      0.353      2.528      0.480

```

END

24.12.92

FIN